UNRAVELING THE PARADOXICAL IMPACTS OF *TOXOPLASMA GONDII* ON DENDRITIC CELL STAT1 SIGNALING

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Toxoplasma gondii is a protozoan parasite of wide geographic and host range. Although many infections in humans are asymptomatic, the potential exists for severe disease. The parasite is highly successful given its ability to establish life-long infections. To achieve this, the parasite must establish a delicate balance between activation and suppression of the host immune response. The cytokine IFNγ is essential for host resistance to the parasite. Therefore, *Toxoplasma* actively manipulates the IFNγ pathway in order to ensure parasite survival.

It has become clear that *Toxoplasma* can evade the host IFNγ response in a variety of cell types. However, the exact mechanism by which this occurs remains unclear. Furthermore, evasion of the IFNγ response has not been studied in the dendritic cell, an immune cell type important during *in vivo* infection. I show that *Toxoplasma* can block STAT1 binding to IFNγ-responsive gene promoters in dendritic cells, preventing transcription. However, STAT1 activation is not impaired. In fact, I show that *Toxoplasma* alone causes STAT1 phosphorylation and nuclear translocation, as well as consensus sequence binding. Phosphorylation and consensus sequence binding occur synergistically with IFNγ. However, the parasite induces formation of aberrant STAT1 DNA-binding complexes.

Further work shows that *Toxoplasma*-induced STAT1 phosphorylation and/or synergy with IFNγ occur independently of the parasite kinases ROP16, ROP18, ROP21 as well as the parasite-triggered host MyD88-, G_iPCR-, and PI3K-dependent signaling pathways. Furthermore, inhibition of STAT1 transcriptional activity occurs in a parasite strain-independent manner. Synergistic levels of phospho-STAT1 correlate with parasite mediated inhibition of IFNγ-induced *Socs1* expression. Yet, the parasite alone induces modest *Socs1* expression in a ROP16-dependent manner, which may participate in regulation of other pathways such as TLR signaling.

From this work it is clear that the impacts of *Toxoplasma* on dendritic cell STAT1 signaling are complex. The parasite induces STAT1 phosphorylation, but how this occurs and whether this STAT1 participates in the transcription of a unique subset of genes remains to be determined. However, it is clear that the parasite can block STAT1 chromatin binding and transcription in dendritic cells in response to IFNγ, permitting evasion of this important host immune response.

BIOGRAPHICAL SKETCH

Anne was born in Washington DC on April 11th, 1983 to Jane and Jack Gordon. She lived in various other places with her parents and three brothers during her childhood, including Pennsylvania, Virginia, upstate New York, Massachusetts, and Delaware. As a child, Anne became very interested in animals. She and her mother raised and showed miniature horses for a period of time. Anne then became interested in horseback riding, at which time she acquired a riding horse and began participating in equine 4-H. Through 4-H, she learned a great deal about horse care in general as well as details of equine anatomy and veterinary care. Anne participated in national 4-H horsebowl and hippology competitions, placing in the top three individually. She also participated in horse judging and public speaking competitions. Her family also had a variety of other animals including dogs, cats, cockatiels, guinea pigs, fish and geese. These early life experiences with animals led Anne to consider pursuing a career in veterinary medicine.

Anne graduated from North Middlesex Regional High School in MA in 2001 as valedictorian of her class. She went on to attend Elizabethtown College (Etown) in Elizabethtown, PA, where she majored in biology and was accepted into the Hershey Foods Honors Program. While at Etown, Anne developed an interest in research and did a project in Diane Bridge's developmental biology lab, cloning and studying the expression of vascular endothelial growth factor isoforms in the cnidarian *Hydra magnipapillata*. During her summers, Anne acquired additional research experience in behavioral pharmacology at AstraZeneca Pharmaceuticals as well as veterinary experience as an assistant at Delmarva Equine Clinic. During her time at Etown, Anne received numerous awards and honors including distinguished student awards for

three straight years. She graduated with a bachelor's of science degree in 2005 *summa cum laude*, with honors in biology.

Given her strong interests in both research and veterinary medicine, Anne decided to apply to the highly competitive dual DVM/PhD program at Cornell University. She was accepted and began the graduate school portion of her program in the Fall of 2005. In the Spring of 2006, she joined Eric Denkers' laboratory, beginning a project on how the protozoan parasite, *Toxoplasma gondii*, modulates STAT1 signaling in murine dendritic cells. She passed the A exam in the summer of 2007 and then transitioned to the veterinary portion of her program, maintaining a presence in the lab when possible. In 2008, she married David Schneider, who had recently obtained his PhD at Cornell in mechanical engineering and subsequently began a career in the systems engineering department. As a veterinary student, Anne excelled academically and won awards in anatomy, physiology, pathology, avian medicine, and small animal medicine & surgery. She participated in various clubs and was president of the pathology club, secretary of the Zoo and Wildlife Society (ZAWS), and a student-elected representative on the curriculum committee. Anne also served as a supervisor at Cornell's wildlife clinic. In addition, she successfully obtained competitive externships at Disney's Animal Kingdom and at the San Diego Zoo's Safari Park that she completed in the Spring of 2011. Anne graduated with her DVM degree in May of 2011, ranked first in her class. At that time, she resumed her PhD training, finishing her thesis work over the next two years. Anne hopes to become involved in teaching the pre-clinical veterinary curriculum in the future. However, she also maintains interests in medicine, pathology, and biomedical research and hopes to develop a career integrating all of these areas.

To my loving and amazing husband, David Schneider

You have remained steadfast by my side throughout this entire journey

I can never thank you enough for all your patience, love, support

and your uncanny ability to bring out the best in me

You are the best partner for life and adventure that I could have ever asked for

and I am so thrilled to begin the next chapter in our lives together

I love you deeply and always will

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I would also like to thank the rest of my committee for all of their efforts in helping me reach this milestone - thank you David Holowka, John Parker, and Kenneth Simpson. I also am grateful to everyone in my lab who I have worked with over the past several years. Barbara Butcher has been instrumental during lab meetings with her great insight, always asking important questions. She has also been there for me when I needed advice or someone to talk to about life in general. I have also worked with the following individuals who have all helped me at some point and been amazing friends: Charlotte Egan, Delbert Abi Abdallah, Sara Cohen, Kirk Maurer, Woraporn Sukhumavasi, Allison Bierly, Jin Leng, and Chiang Lee. Thank you also to the lab technician, Mozammal Hossain, for his technical support. I have also been blessed with amazing officemates who have been great sources of support. Thanks also to the rest of the Microbiology & Immunology department for all their support, friendship, advice, research insight, and assistance over the years. I would especially like to thank Dwight Bowman who has been my veterinary school advisor and also a great person to go to for advice and friendship.

Thanks also go out to the dual DVM/PhD program, including members of the oversight committee as well as fellow program students. I was so lucky to have such an amazing opportunity to pursue both degrees while also being supported financially. Despite the difficulties that arose over the years, everyone remained supportive and encouraging.

My family has also been instrumental in getting me to this point. I would like to thank my parents, Jane and Jack Gordon, as well as my brothers John, Paul, and Bill, for all of their love and support. My parents have been especially amazing in terms of encouraging me to pursue my education and providing me with financial support to do so. I would not have made it here without their sacrifices, love, and faith in me. Thank you guys for everything! Of course, I am also immensely grateful to my husband David Schneider, as described in the dedication. I love you babe! Thanks go out to him for keeping me sane throughout this intense 8 year dual degree program. He has been a great reminder to enjoy life, to strive to be a better person, and to seek out adventure! Thanks also to Dave's family – Carol, Bob, and Andrea Schneider. They have always been there for me, going the extra mile to support me in any they can. Their kindness and efforts to help me have been very much appreciated. Thanks also to all my Etown friends and faculty who I continue to stay in touch with and who have supported me all along. I am also grateful to my pets for the joy they give me, especially my cockatiels Captain Puff and Archie.

Finally, I greatly appreciate and acknowledge the sacrifice of the animals that was required in order for me to perform my experiments and complete my PhD degree. If it weren't for laboratory animals, we couldn't achieve many of the amazing things we do with biomedical research. Thanks to all those animals that lost their lives in order to make our lives better.

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LIST OF ABBREVIATIONS

AIDS, acquired immunodeficiency syndrome
AMA1, apical membrane antigen 1
ATF6β, activating transcription factor 6 beta
ATP, adenosine triphosphate
BAF, Brahma-related gene 1/Brahma 1-associated factor
BMDC, bone marrow-derived dendritic cell
BMDM, bone marrow-derived macrophage
BRG, Brahma-related gene
BSA, bovine serum albumin
BTK, Bruton's tyrosine kinase
CaMKII, calcium/calmodulin-dependent protein kinase II
CBP, CREB-binding protein
CD, cluster of differentiation
cDC, conventional DC
cDNA, complementary DNA
ChIP, chromatin immunoprecipitation
CIITA, class II transactivator
CXC, chemokine C-X-C motif
CR, chemokine receptor
CREB, cyclic adenosine monophosphate response element-binding protein
DC, dendritic cell
DMEM, Dulbecco's modified eagle medium
DNA, deoxyribonucleic acid
DOT1L, disruptor of telomeric silencing-like histone H3 methyltransferase
DTT, dithiothreitol
EDTA, ethylenediaminetetraacetic acid
ELISA, enzyme-linked immunosorbent assay
EMSA, electrophoretic mobility shift assay
ERK, extracellular signal-regulated kinase
FACS, fluorescence-activated cell sorting
FITC, fluorescein isothiocyanate
GAF, gamma-activated factor
GAPDH, glyceraldehyde 3-phosphate dehydrogenase
GAS, interferon gamma-activated sequence
GBP, guanylate-binding protein
G_iPCR, G inhibitory protein-coupled receptor
GM-CSF, granulocyte/monocyte-colony-stimulating factor
GRA, dense granule
GTPase, guanosine triphosphatase
HIV, human immunodeficiency virus
IB, immunoblot
IDO, indoleamine 2,3- dioxygenase

IFA, immunofluorescence assay
IFN, interferon
IFNγR, interferon gamma receptor
Ig, immunoglobulin
IL, interleukin
iNOS, inducible nitric oxide synthase
IP, immunoprecipitation
IRAK, interleukin-1 receptor-associated kinase
IRF, interferon regulatory factor
IRG, immunity-related GTPase
IκB, inhibitor of NF-κB
JAK, Janus kinase
JNK/SAPK, c-Jun N-terminal kinase/stress-activated protein kinase
KCl, potassium chloride
KO, knock-out
LD50, lethal dose for 50% of the population
LPS, lipopolysaccharide
Ly6C, lymphocyte antigen 6C
M1, classically activated macrophage
M2, alternatively activated macrophage
Mal/TIRAP, MyD88 adaptor-like/toll-interleukin 1 receptor domain containing adaptor protein
MAPK, mitogen-activated protein kinase
Mcm, minichromosome maintenance
Med, medium
MHC, major histocompatibility complex
MIC, microneme
MIG, monokine induced by interferon gamma
moDC, monocyte-derived DC
MOI, multiplicity of infection
MyD88, myeloid differentiation primary response 88
NAS, nucleic acid-sensing
NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells
NK, natural killer
Nmi, N-myc and STATs interactor
NO, nitric oxide
P, phosphate group
PARP, poly ADP ribose polymerase
PBS, phosphate-buffered saline
PCR, polymerase chain reaction
pDC, plasmacytoid DC
PI3K, phosphoinositide 3-kianse
PIAS, protein inhibitor of activated STAT
PKB, protein kinase B
PKC, protein kinase C
PMSF, phenylmethylsulfonyl fluoride
Poly I:C, polyinosinic:polycytidylic acid

PRF, profilin
PTP, protein tyrosine phosphatase
Ptx, pertussis toxin
PV, parasitophorous vacuole
PVM, parasitophorous vacuole membrane
RNA, ribonucleic acid
ROI, reactive oxygen intermediate
RON, rhoptry neck
ROP, rhoptry bulb
SAG1, surface antigen 1
SCID, severe combined immunodeficiency
SDS-PAGE, sodium dodecyl sulfate-polyacrylamide agarose gel electrophoresis
SH2, Src homology 2
SHP, SH2 domain-containing phosphatase
SOCS, suppressor of cytokine signaling
Sp1, specificity protein 1
STAT, signal transducer and activator of transcription
SUMO, small ubiquitin-like modifier
TBST, tris-buffered saline with Tween-20
TC45, T cell phosphatase 45
Tg, *Toxoplasma gondii*
TgCyc18, *Toxoplasma gondii* cyclophilin 18
TGF, transforming growth factor
Th1, T helper cell type 1
TipDC, TNF and iNOS-producing DC
TLR, toll-like receptor
TNF, tumor necrosis factor
TRAF6, TNF receptor-associated factor 6
Treg, T regulatory cell
U-STAT, unphosphorylated signal transducer and activator of transcription
WT, wild type
Wtm, wortmannin

LIST OF SYMBOLS

α, alpha
β, beta
©, copyright
°, degree
δ, delta (lower case)
Δ, delta (upper case)
κ, kappa
μ, mu or micro

CHAPTER 1

Introduction

TOXOPLASMOSIS

Etiology and history

Toxoplasma gondii is the protozoan responsible for the disease of humans and animals known as toxoplasmosis. The parasite was initially discovered in 1908 in Tunis by Nicolle and Manceaux in a North African hamster-like rodent known as the gundi (*Ctenodactylus gundi*). Around the same time, Splendore identified the same parasite in a rabbit, but mistook it for *Leishmania*. Nicolle and Manceaux named the parasite based on its form (*Toxo* = arc or bow; *plasma* = life) and host (the gundi, which they mistakenly identified as gondi initially) (1-3). One of the first cases of acquired human toxoplasmosis was identified by Sabin and colleagues in a young boy who had experienced head trauma from a baseball bat and coincidentally died 30 days later as a result of a *Toxoplasma* infection in 1937. The name of a parasite strain commonly used today in research laboratories, RH, was derived from the boy's initials (2). Around the same time, the first documented case of congenital toxoplasmosis resulting in a death of a newborn in 1938 was reported by Wolf, Cowen and Paige (4). Since then, the parasite has been identified world-wide in virtually all warm-blooded vertebrates, with all isolates belonging to a single species (2).

Toxoplasma belongs to the phylum Apicomplexa, which contains ~6000 organisms to date, most of which are obligate parasites at some point during their complex life cycles. Some, like *Toxoplasma*, have been identified as pathogenic to humans and/or animals and cause other important diseases such as malaria (*Plasmodium* spp), babesiosis (*Babesia* spp), coccidiosis (*Eimeria* spp), cryptosporidiosis (*Cryptosporidium* spp), and theileriosis (*Theileria* spp) (5). The Apicomplexans are named for the apical complex, which consists of various secretory organelles (micronemes, rhoptries, dense granules), the conoid (involved in invasion), and a microtubule

organizing center known as the apical polar ring (6). In addition, many possess a non-photosynthetic plastid known as the apicoplast, which is essential for *Toxoplasma* survival, presumably due to its role in fatty acid and isoprenoid synthesis (7-11).

Like most other Apicomplexans, *Toxoplasma* relies on substrate-dependent gliding motility to travel across biological barriers and to invade and egress from host cells. A macromolecular complex known as the "glideosome" facilitates movement, consisting of microneme-derived secreted adhesive proteins that are released apically and then translocated to the posterior pole of the parasite (12). This complex is powered by the parasite's actin-myosin system anchored in the inner membrane complex (13, 14). Active invasion and establishment of the parasitophorous vacuole within the host cell requires gliding motility as well as sequential secretion of the apical organelles known as micronemes, rhoptries, and later, dense granules (15) (Figure 1.1). In addition to motility, microneme proteins facilitate attachment to the host cell. As *Toxoplasma* invades, a "moving junction" is formed with the host cell, which consists of a microneme protein (AMA1) on the parasite surface engaged with a rhoptry neck proteins (RONs) secreted into the host cell membrane (12, 16). The moving junction slides over the parasite as it invades, acting as a sieve that removes the majority of host proteins in order to establish a non-fusogenic parasitophorous vacuole (PV) (12, 17-19). Other rhoptry proteins (ROPs) help to further establish the PV or serve as virulence factors, modulating the host response to infection (20). The PV is further modified as dense granule proteins are continuously secreted to maintain the PV and assist in nutrient acquisition (21). After several rounds of division, tachyzoites actively egress from the cell in a calcium-dependent manner very similar to the process of invasion (22).

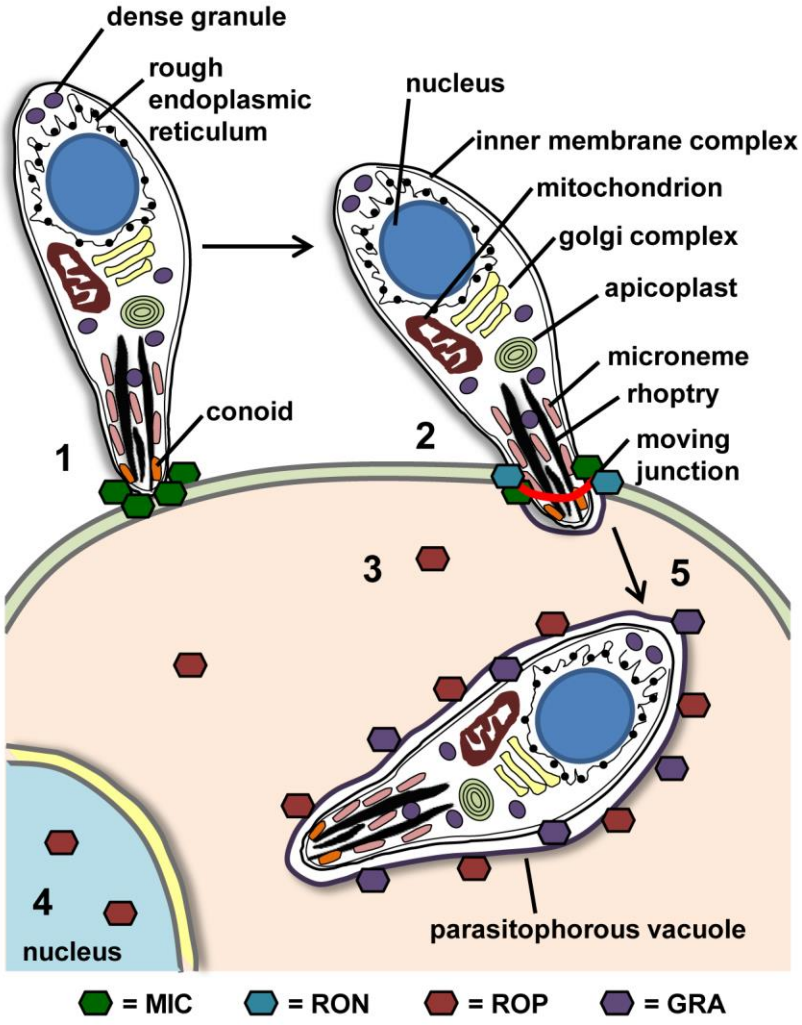


Figure 1.1. Host cell invasion and establishment of the parasitophorous vacuole by *Toxoplasma gondii*. Upon contact with a target host cell, the parasite initiates invasion by secreting microneme proteins (MIC) that serve as attachment factors (1). Shortly after attachment, the "moving junction" (red line) is established between the parasite and the host cell, consisting of the microneme protein AMA1 on the parasite surface interacting with rhoptry neck proteins (RON) secreted into the host plasma membrane (2). Also during this time, rhoptry bulb proteins (ROP) are released. Many are inserted into the parasitophorous vacuole membrane but others can be found in the host cell cytosol (3) or even in the nucleus (4). Passage of the parasite through the moving junction permits establishment of the non-fusogenic parasitophorous vacuole within the host cell cytoplasm (5). At this time, the parasite further modifies the parasitophorous vacuole by secreting dense granule proteins (GRA) into the vacuole membrane and lumen (5). Components of the ultrastructure of the parasite are indicated as shown.

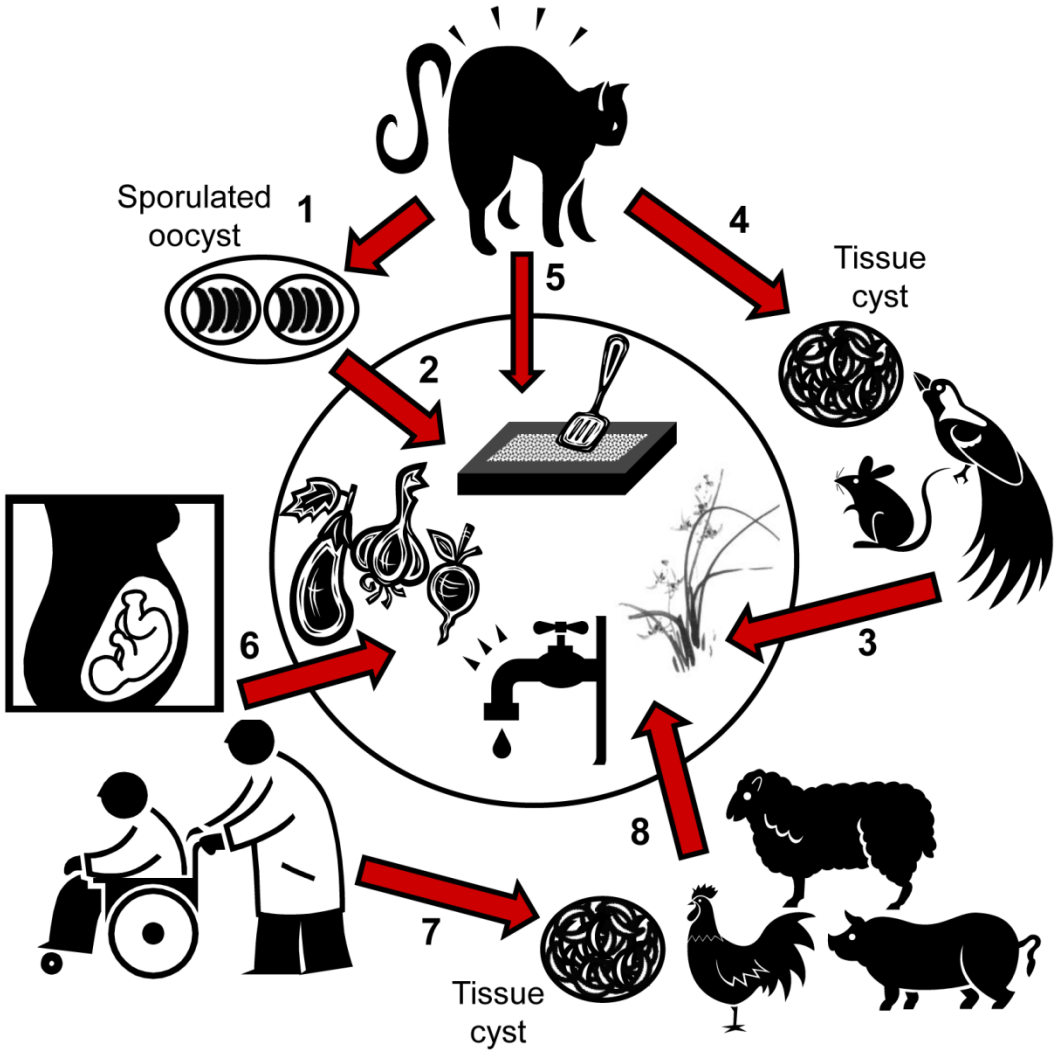
Life cycle, population structure, and transmission

Toxoplasma possesses a complicated life cycle, with both sexual and asexual stages, as reviewed by Dubey (23), summarized here, and depicted in Figure 1.2. There are three infectious forms of the parasite, including the rapidly dividing tachyzoite, the slowly-dividing bradyzoite (found within tissue cysts), and the environmentally resistant stage, the sporozoite (found within sporulated oocysts). The sole definitive host for the parasite is the cat. A feline is most efficiently infected through the ingestion of tissue cysts in an intermediate host (often a rodent or bird), although sporulated oocysts or tachyzoites may also be a source. The cyst wall is degraded in the gastric environment, releasing the bradyzoites which subsequently invade the cat's intestinal epithelial cells. Conversion to tachyzoites results in multiple rounds of asexual proliferation by endodyogeny, allowing for dissemination of the parasite throughout the cat, as in intermediate hosts. In addition, the parasite undergoes an intensive enteroepithelial cycle within the cat's intestine, forming schizonts of five types, A-E. At the end of schizogony, merozoites are released to form male and female gametes, which in turn fuse to form a zygote enclosed within a wall (the oocyst). Epithelial cells rupture to release the mature oocysts, which are subsequently transmitted into the environment through the feces. It takes approximately 3-7 days after ingestion of tissue cysts for a cat to shed oocysts. Shedding can persist for up to 20 days, with up to 100 million oocysts potentially released (23).

Oocysts become infective approximately 1-5 days after being passed in the feces, once the process of sporogony has taken place. The completely sporulated oocyst consists of 2 sporocysts containing 4 haploid sporozoites each. Sporulated oocysts are environmentally resistant, with the ability to persist for years in soil and water under optimal conditions (24-26) as well as being

resistant to some chemical and physical methods of inactivation (27, 28). A wide range of warm-blooded intermediate hosts, including humans, can ingest the oocysts present in contaminated food and water. Upon ingestion, released sporozoites invade the intestinal epithelium, transform into tachyzoites, and disseminate from the intestine to the rest of the host's tissues. Tachyzoites dominate during the acute phase of infection and can invade nearly all nucleated cells. After a number of divisions and under pressure from the host's immune system, tachyzoites convert to the slowly dividing bradyzoite stage to establish chronic infection. A cyst wall forms around multiple bradyzoites, generating tissue cysts. Although cysts can form in various tissue types, they are most abundant within long-lived neural and muscular cells. There they persist for the lifetime of the host. The life cycle is complete when the intermediate host containing tissue cysts is ingested by a feline. However, *Toxoplasma* does not require the sexual phase of the life cycle to reproduce (unlike other Coccidians) and thus can propagate asexually between intermediate hosts via ingestion of tissue cysts (23).

Figure 1.2. The transmission and life cycle of *Toxoplasma gondii*. Felines serve as the sole definitive host of the parasite, shedding oocysts in the feces. After a few days, the oocysts undergo sporulation and become infective (1). In addition to being present in cat feces, sporulated oocysts can also contaminate food and water supplies for people as well as other animals (2). Small prey species such as rodents and birds become infected primarily through ingestion of oocyst-contaminated food or water (3). The parasite rapidly replicates within the host as tachyzoites initially, but then undergoes conversion to the chronic stage, forming cysts in various tissues but particularly muscle and nervous tissue. The cat becomes reinfected by ingesting prey species containing the tissue cysts (4), although they can also ingest oocyst-contaminated material to a lesser extent (5). Humans become infected by ingesting food or water contaminated with sporulated oocysts from cat feces (6) or by ingesting undercooked meat containing tissue cysts (7). Undercooked meat is derived from animals serving as intermediate hosts that became infected through ingestion of food or water contaminated with sporulated oocysts (8). Vertical transmission from an infected mother to the fetus can also occur and result in devastating consequences for the fetus. Organ transplantation can also serve as a source if donors are infected. People who are chronically infected and subsequently become immunocompromised, as with HIV/AIDS patients and those receiving immunosuppressive drug therapy, are also susceptible to developing toxoplasmosis.


Sporulated
oocyst
1
2
3
4
5
6
7
8
Tissue
cyst
Tissue
cyst

The unique ability to propagate asexually may explain why three main clonal lineages of the parasite dominate infections of people and domestic animals in North America and Europe (29, 30). A fourth clonal type was also recently identified in North America, common in wildlife (31, 32). The *Toxoplasma* population likely underwent a bottleneck ~10,000 years ago, allowing the four clonal lineages to rapidly expand their host ranges since that time (33). In contrast, strains in South America show much greater diversity within and between groups, indicating that more sexual recombination has taken place in that geographic area (34, 35). The three clonal lineages initially identified are designated type I, type II and type III and differ from one another genetically by only 1-2% (29). Despite this high similarity, they exhibit differences in terms of virulence. Type I parasites are uniformly lethal in mice (LD50=1), whereas type II and III strains are much less virulent (LD50=10^3-10^5) and able to establish chronic infection (36). Several polymorphic effector proteins secreted by the parasite have been identified as key virulence factors, accounting in part for the observed strain differences (37). Less is known about how strain type affects disease in humans and other animals. Type II strains are most commonly identified in people in North American and Europe with toxoplasmosis while type III strains predominate in other animals and do not appear to cause much disease (38). Type I strains appear to dominate in severe congenital infections (38), and are also implicated, along with atypical strains, in some cases of severe ocular toxoplasmosis in non-immunosuppressed patients (39, 40). Cases of severe ocular toxoplasmosis in patients who are otherwise healthy are common with South American isolates (41, 42). Thus, knowledge of mechanisms by which different strains cause disease may prove useful in future therapies for human cases.

Knowledge of the life cycle is essential for identifying means by which people can become infected (Figure 1.2). One primary area of concern is food safety, as people are most apt to acquire *Toxoplasma* via the ingestion of contaminated food and water supplies. In fact, a recent study published by the Centers for Disease Control ranked toxoplasmosis as the 2nd leading cause of foodborne-related deaths and 4th highest in terms of causing hospitalizations (43). People acquire foodborne toxoplasmosis primarily through ingestion of undercooked meat containing tissue cysts or via ingestion of food or water contaminated with oocysts (44). The parasite can also be transmitted across the placenta to the fetus in pregnant women who are infected for the first time during pregnancy (45). Vertical transmission may also play a role in maintaining infections in other animal species, such as sheep (46). The clinical implications of *Toxoplasma* infection in humans and other animals are addressed in the next section.

Clinical aspects of Toxoplasmosis

It is estimated that *Toxoplasma* infects approximately 25-30% of the world's human population (47), highlighting the widespread success of this parasite. In the United States, prevalence ranges from 10-20%, whereas seroprevalence exceeds 60% in parts of South America, Oceania, and Africa (48). For most immunocompetent individuals, the infection is asymptomatic or presents with mild flu-like symptoms during the acute phase. However, serious and potentially fatal consequences can occur in patients with compromised immune systems or who are pregnant (47). Immunosuppression most commonly results in disease by allowing cyst rupture and resultant reactivation of a chronic infection. In this situation, the strain of parasite becomes less important as the host immune status largely dictates the response to infection (49). Those at risk include HIV/AIDs patients, organ transplant recipients, and those undergoing chemotherapy for

cancer or immune-mediated disease. Patients infected with human immunodeficiency virus (HIV) become more likely to develop toxoplasmosis as $CD4^+$ T cells drop below 100 cells/μl, which can be minimized through effective anti-retroviral drug therapy (50). Toxoplasmic encephalitis is the primary manifestation of the disease in immunocompromised individuals. Symptoms are variable and may include headache, fever, dementia, ataxia, hemiparesis, memory loss, seizures, coma, and death. Other organs may also be affected and in particular the lungs (pneumonia), heart (myocarditis), and eyes (retinochoroiditis) (51). Disseminated or pulmonary toxoplasmosis is more commonly seen in transplant recipients (52).

Congenital infections resulting from vertical transmission of *Toxoplasma* are another source of concern. Usually, vertical transmission is only thought to occur in women who are infected with the parasite for the first time during pregnancy, but exceptions do occur and may be related to parasite strain type in such cases (53). The likelihood of successful transmission and the outcome for the fetus depend on the time during pregnancy at which the woman is infected. In the first trimester, the frequency of transmission is only 10%; however, the consequences for the fetus are severe, resulting in severe abnormalities or abortion. By the third trimester, transmission frequency is high at 60-70% whereas 80% of infected neonates are asymptomatic at the time of birth (54, 55). However, ocular lesions such as retinochoroiditis can develop as late as the second or third decade of life if infants are not treated sufficiently (56). Manifestations of congenital toxoplasmosis are varied and can mimic other diseases but may include hydrocephalus, intracranial calcifications, retinochoroiditis and other ocular abnormalities, mental retardation, and/or seizures (57).

Immunocompetent and otherwise healthy individuals can also develop disease secondary to *Toxoplasma* infection. Usually this consists only of mild, non-specific clinical signs such as fever and lymphadenopathy. However, cases of severe ocular toxoplasmosis have also occurred in the absence of immunosuppression or history of congenital exposure. Many studies have shown higher incidences and severities of retinochoroiditis in Brazil with both primary infections in immunocompetent individuals as well as in those congenitally-infected (58). These cases are most often linked to atypical strain types originating from South America or Africa (39, 59, 60). Therefore, a better understanding of virulence mechanisms by these strains may be beneficial in future therapies and preventative measures.

In addition to causing disease in humans, *Toxoplasma* can cause serious economic loss in terms of livestock infections. In particular, sheep and goats have been severely affected with outbreaks resulting in reproductive losses such as embryonic loss/resorptions, abortions, fetal mummifications, stillbirths, and neonatal deaths (61). Illness in pigs in countries like Japan has also been reported with cases of abortion, myocarditis, pneumonia, and encephalitis (62). Devastating consequences can also occur for certain wildlife and zoo animal species infected with the parasite. Kangaroos/wallabies (63), new world monkeys (64), and marine mammals are particularly susceptible (65).

Diagnosis, treatment, and prevention

In cases where *Toxoplasma* exposure is suspected, serology testing is most frequently performed. Most commonly, testing is performed to detect both IgM and IgG. IgM is produced as early as the first week of infection, reaching a plateau at 1 month and then rapidly declining to minimal

or undetectable levels after about 6 months (49). Depending on the test used, IgG can be detected 1-3 weeks after the IgM peak, reaching a plateau at 2-3 months and then decreasing rapidly to a lifelong residual titer (49). There are multiple tests available to detect antibody production including the Sabin-Feldman dye test, ELISA, IFA, and various agglutination assays; however, most laboratories today use ELISAs for parasite-specific IgM and IgG to look for evidence of acute and chronic infection, respectively (49). However, given that IgM titers can persist for 1-2 years in some cases, IgG avidity testing can also be employed to rule out a recent infection within the past four months (66). PCR for parasite specific antigens can also be performed on fluid or tissue samples in the case of congenital infections or in immuno-compromised patients exhibiting disease symptoms. The *Toxoplasma* B1 gene has been most consistently used in PCR detection, but recently a test for the REP-529 has been developed, which is more sensitive given that this gene is repeated 200-300 times versus 35 times for the B1 gene (67). Immunohistochemistry or Giemsa staining may also be performed on tissue samples for a diagnosis. If desired, parasite strains can be isolated and typed following inoculation into a mouse (bioassay) or cell culture. Ocular toxoplasmosis is most commonly diagnosed based on lesions found on ophthalmological exam in combination with serology (49).

Treatment usually consists of a long-term course of potentiated sulfa antibiotics. The most effective standard course of therapy combines pyrimethamine (50-100 mg) and sulfadiazine (4-8 mg) daily, which act synergistically to inhibit tachyzoite folic acid synthesis (68). However, the chronic cyst form of the parasite is unaffected. Folinic acid (10 mg/day) is often added to combat bone-marrow suppression. Clinical improvement is seen in 70-90% of patients. However, adverse side effects occur 20-50% of the time and include a rash, anemia,

thrombocytopenia, neutropenia, and allergic reaction (69). In patients that cannot tolerate a sulfa drug, clindamycin may be used instead (70). Multiple alternative drugs have been tested in small trials such as atovaquone, various macrolide antibiotics, doxycycline, and others, but none have been shown to be more effective than standard therapy (68). The macrolide spiramycin is commonly used to treat at risk pregnant women during the first 18 weeks of gestation, as this antibiotic is thought to decrease the likelihood of vertical transmission and is not teratogenic (71). However, if fetal infection is confirmed at 18 weeks or later when amniocentesis is typically performed, the woman is switched to the standard therapy as spiramycin is unable to cross the placenta. Sulfa drugs cannot be administered during the first trimester due to risk of teratogenesis (71). Relapses of toxoplasmosis following therapy are common, occurring 40-80% of the time. Future advances in immunotherapy may help to prevent relapses in the immunocompromised, but additional treatments that target the cyst stage of the parasite will be needed if a sterile cure is to be obtained.

Prevention of toxoplasmosis relies heavily on avoiding contact with viable parasite tissue cysts and oocysts and is summarized as follows (49). Pregnant women should be especially careful given the risk to the unborn child. People should wash their hands thoroughly after handling raw meat, and ideally cook all meat to well done or at least freeze meat ahead of time for at least two weeks to eliminate viable tissue cysts. Meat obtained from wild game or organically raised production systems provides the most risk. Soil can become contaminated with oocysts shed by felids in the environment, and thus it is wise to wash all fruits and vegetables thoroughly prior to eating them raw. It is also important to wear gloves while gardening to minimize soil contact. Sources of unfiltered water should be avoided as much as possible since water transmission of

oocysts has caused toxoplasmosis outbreaks. House cats are not considered a major cause of infection in people, given that cats generally only shed oocysts once in their lives for about 2 weeks, typically as kittens. However, pregnant women should still take precautions to wash their hands after cat contact, avoid contact with cat litter, and have someone else change the litter daily since oocysts take 2-3 days to become infectious in the environment. Keeping cats indoors to minimize exposure to wild prey and feeding only commercial cat foods is also advised. No vaccines are currently available for people. A variety of vaccine strategies have been attempted in rodent models, but are generally unsuccessful in inducing sterile immunity upon subsequent challenge (72). A live attenuated vaccine (S48) is available, however, to reduce *Toxoplasma*-induced abortion in sheep (73).

Toxoplasmosis is a widespread disease of serious concern to pregnant women and immuno-compromised individuals, yet treatment measures pose risks of side effects and relapse while no effective vaccine exists to protect those at highest risk. Therefore, a better understanding of the host's immune response to the parasite is critical to future rational design of more effective treatment and prevention measures.

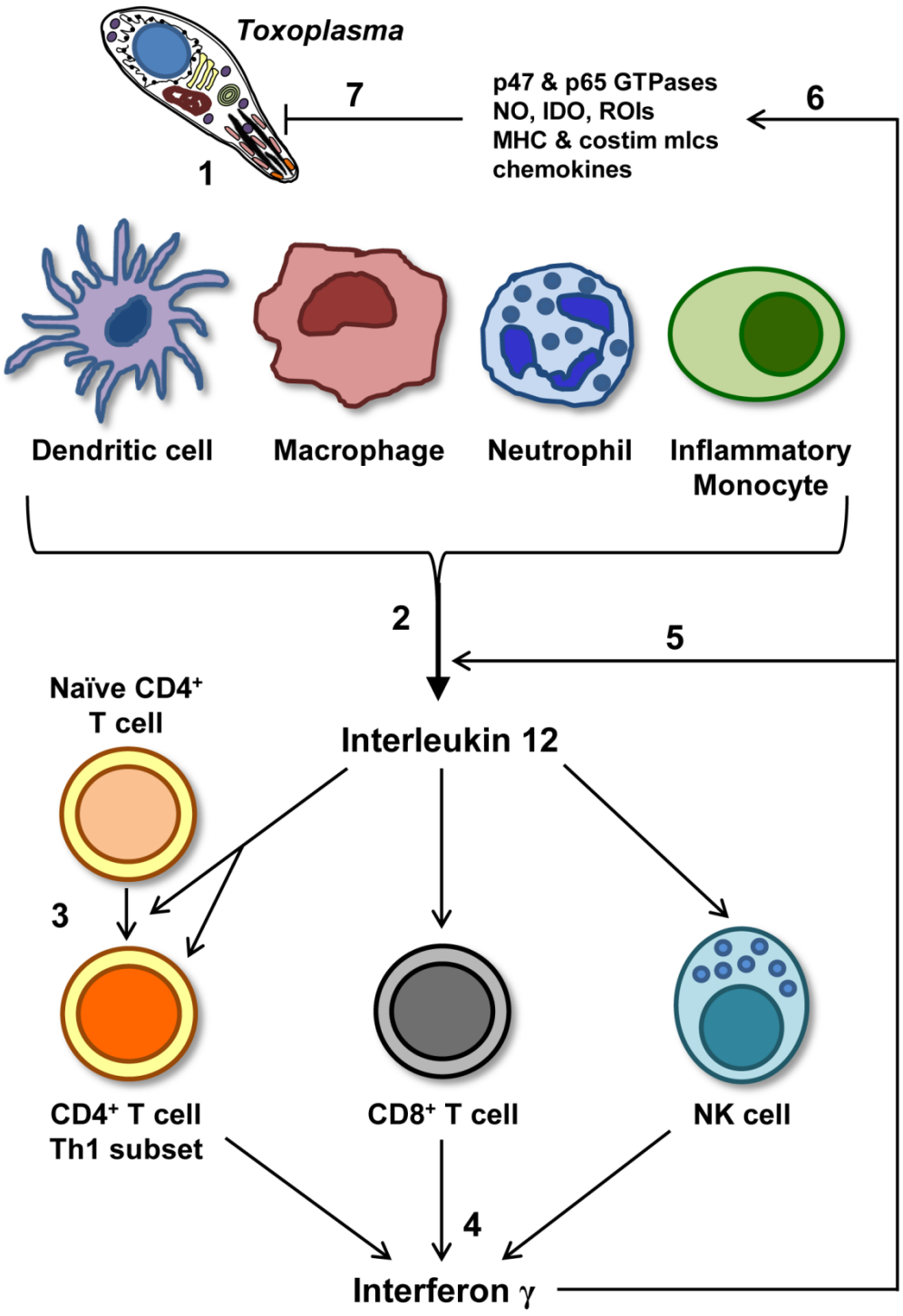
THE IMMUNE RESPONSE TO *TOXOPLASMA GONDII*

A robust host immune response is necessary to mitigate the impacts of *Toxoplasma* infection. The human immune response to the parasite has not been well characterized, as most people are asymptomatic or develop disease symptoms long after initial infection. Thus, the focus here will be on the extensively studied murine immune response.

Th1-based immunity during acute infection

Survival of acute in vivo *Toxoplasma* infection in mice relies upon induction of a strong Th1 cell-mediated immune response (Figure 1.3). Innate immune cells including dendritic cells, macrophages, inflammatory monocytes and neutrophils play key roles in the Th1 response by secreting the pro-inflammatory cytokine interleukin 12 (IL12) in response to the parasite (74). This cytokine induces differentiation of $CD4^+$ T cells into the Th1 subset, which, along with $CD8^+$ T cells and NK cells, produce the pro-inflammatory cytokine interferon gamma (IFNγ) (75, 76). IFNγ in turn acts back on the innate immune cells, inducing further IL12 production along with various antimicrobial effector mechanisms that serve to control the parasite (74). The importance of both Th1 cytokines is highlighted by studies where depletion or genetic ablation of IL-12 or IFNγ renders mice acutely susceptible to *Toxoplasma* infection (77-79).

Figure 1.3. The Th1 immune response to *Toxoplasma gondii*. Cells of innate immunity, including dendritic cells, macrophages, neutrophils, and inflammatory monocytes, sense the presence of tachyzoite antigens through pattern recognition receptors (1). Recognition results in innate immune cell activation, followed by secretion of the pro-inflammatory cytokine interleukin 12 (IL12) (2). IL12 triggers differentiation of naïve $CD4^+$ T cells into the Th1 subset (3), while also promoting secretion of IFNγ from Th1 T cells, $CD8^+$ T cells, and NK cells (4). IFNγ in turn acts back on innate immune cells to induce further IL12 production (5). In addition, IFNγ enhances antigen presentation, chemokine production, and activation of various antimicrobial effector mechanisms in innate immune cells (6). Through these activities, the end result of the Th1 immune response is destruction of the parasite (7).


Toxoplasma
7
p47 & p65 GTPases
NO, IDO, ROIs
MHC & costim mlcs
chemokines
6
1
Dendritic cell
Macrophage
Neutrophil
Inflammatory
Monocyte
2
5
Naïve CD4+
T cell
Interleukin 12
3
CD4+ T cell
Th1 subset
CD8+ T cell
NK cell
4
Interferon γ

Innate immune responses are sufficient during the early phase of infection, as studies in infected SCID mice (which are deficient in B and T cells) demonstrate (80). The major early producer of IFNγ is the NK cell (81). However, after about 20 days of infection, SCID mice die unless reconstituted with T cells, highlighting the importance of T cells during the chronic phase (80). Both $CD4^+$ and $CD8^+$ T cells are required to prevent reactivation of a chronic *Toxoplasma* infection in mice, in large part due to T cell-mediated IFNγ secretion (82). Endogenous IFNγ is required to prevent toxoplasmic encephalitis development in infected mice (83). In addition, both T cell types act synergistically to provide required IFNγ-dependent protective immunity upon challenge in an attenuated *Toxoplasma* vaccine model (84). In addition to IFNγ production, $CD4^+$ T cells produce IL2 which serves to amplify $CD8^+$ T cell IFNγ production (84), while $CD8^+$ T cells also induce perforin-mediated cytolysis that contributes to resistance to toxoplasmic encephalitis (85). In addition to IFNγ and perforin, other contributors to resistance during chronic *Toxoplasma* infection include TNFα (86) and inducible nitric oxide synthase (87). B cells and antibody production are not thought to be major contributors to acute resistance to *Toxoplasma*, as transfer of serum from infected mice to uninfected animals is not sufficient for protection against parasite challenge. However, μMT mice that lack B cells are susceptible during the chronic phase, suggesting that B cells may indeed play some role at later time points (88). The B cell requirement may depend on cytokine secreting effector B cell subsets rather than antibody production or antigen presentation (89), but further work must be done to address this.

The toxoplasmacidal properties of IFNγ

IFNγ is widely considered the primary mediator of host resistance to *Toxoplasma* infection (77). This cytokine is critical in the control of intracellular parasite replication under *in vitro* and *in vivo* conditions in both mice and humans (77, 78, 90-92). IFNγ mediates this effect through various methods including reactive oxygen intermediates (ROIs), nitric oxide (NO), iron deprivation, tryptophan starvation, immunity-related p47 GTPases (IRGs), and guanylate-binding proteins (GBPs) (74, 93). ROIs include various free radicals that damage cell constituents. Although they have been shown to be toxoplasmacidal in human macrophages (92), *Toxoplasma*-infected murine macrophages are relatively ROI resistant (94) and mice lacking inducible oxidative burst capacity (p47 $phox^{-/-}$) survive acute and chronic *in vivo* infection (95). IFNγ-induced NO is synthesized by the inducible nitric oxide synthase (iNOS) enzyme and kills parasites directly by targeting essential mitochondrial and nuclear enzymes of the tachyzoite (96). TNFα produced by activated macrophages synergizes with IFNγ to induce NO synthesis (97). Although iNOS activity is dispensable during acute infection in mice, it is required to control the chronic phase of infection within the brain (87). This is likely in part due to the role NO plays in promoting conversion of the tachyzoite into the chronic, cyst-contained bradyzoite (98). Iron is a crucial co-factor in metabolic processes for many pathogens, *Toxoplasma* included. Enterocytes in the intestines have been shown to sequester iron in response to IFNγ, thereby limiting parasite replication (99). IFNγ can also induce expression of indoleamine-2,3-dioxygenase (IDO), an enzyme that degrades the amino acid tryptophan which *Toxoplasma* must scavenge from the host in order to replicate. IDO contributes to *Toxoplasma* control in human fibroblasts (100), glioblastoma cells (101), brain endothelial cells (102), and retinal pigment epithelium (103), but does not appear to be important in human macrophages or

murine fibroblasts, astrocytes, and macrophages (102). IDO may still play some role in mice in the control of *Toxoplasma* infection, given evidence of enzyme activity and expression in the lungs and brain of infected mice (104). However, there is strong evidence that the families of immunity related GTPases play the key role in IFNγ-mediated resistance to *Toxoplasma* (105).

The p47 GTPases localize to the endoplasmic reticulum and Golgi apparatus of a variety of cell types in mice (106). They are dramatically upregulated in response to IFNγ stimulation, and are of particular importance for pathogen clearance in innate immune cells such as macrophages and DCs (107). There are 23 known members in mice but only 2 in humans, which also lack IFNγ response elements and thus are not part of the IFNγ resistance program (108). The following four play a role in the resistance to *Toxoplasma* infection *in vivo* in mice: IRGM1 (LRG-47) (109), IRGD (IRG-47) (109), IRGM3 (IGTP) (110), and IRGA6 (IIGP1) (111). Upon infection of an IFNγ-activated cell, the IRGs are recruited to and mediate destruction of the parasitophorous vacuole membrane (PVM), resulting in eventual lysosome-mediated degradation of the parasite (112, 113). Additional IRGs have been shown to traffic to the PVM, including IRGB6 (TGTP), IRGM2 (GTPI), and IRGB10 (114, 115). Members of the IFNγ-inducible p65 family of GTPases known as GBPs are found in 13 copies in mice and 7 in copies in humans (116). They have also been shown to accumulate at the PVM in *Toxoplasma* infected murine cells (117). Furthermore, mice deficient in a cluster of 6 *Gbp* genes are susceptible to acute *Toxoplasma* infection *in vivo (118).* Macrophages from these mice are defective in IFNγ-dependent control of parasite growth and are unable to recruit the p47 GTPases IRGB6 and IRGB10 to the PVM, suggesting that GBPs may function in part through regulation of IRG recruitment (118). In addition, Gbp2 and Gbp1 have recently been shown to play important roles

during acute *in vivo* infection with *Toxoplasma* (119, 120). What role GBPs may play in the IFNγ-mediated resistance to *Toxoplasma* in human cells remains to be determined.

Dendritic cell function and importance during *Toxoplasma* infection

Dendritic cells (DC) are particularly important in the Th1-mediated immune response to the parasite. DCs serve as an important bridge between innate and adaptive arms of the immune response. As part of innate immunity, DCs survey the tissues for pathogens, recognizing pathogen-derived danger signals through pattern recognition receptors such as toll-like receptors (TLRs) (121). In response, DCs migrate to secondary lymphoid tissue where they up-regulate MHC and co-stimulatory molecules and secrete IL12 or other cytokines that serve to initiate adaptive immune responses carried out by lymphocytes (122, 123). Other DCs remain resident in lymphoid tissues where they scan the blood for antigen and then present to T cells locally (123).

Dendritic cells comprise a heterogeneous population with numerous subsets that may be classified based on cell surface marker expression, location in the body, and type of immune function (124). Mature DCs express the integrin CD11c to varying degrees and can be broadly divided into two main categories: conventional DCs and non-conventional DCs (125). Conventional DCs (cDCs) predominate in the steady state and can be divided into migratory and lymphoid-resident DC populations. Migratory DCs can be found in organs such as the intestine, skin, lung, liver, and kidney and will migrate to secondary lymphoid tissues upon activation. They can be further distinguished based on CD103 and CD11b expression (125). Lymphoid resident DCs can be found in the thymus, lymph nodes, and spleen, do not migrate, and can be

further classified on the basis of CD4 and CD8 expression. $CD8^+$ DCs in mice are highly efficient in the cross-presentation of exogenous antigen to $CD8^+$ T cells and are of particular importance in the immune response to intracellular pathogens (126). $CD4^+$ DCs on the other hand are unable to cross-present efficiently *in vivo* but may be better at MHCII-restricted antigen presentation to $CD4^+$ T cells (127).

Non-conventional DCs can also be found to some extent under steady state conditions but are primarily induced under inflammatory conditions and include the plasmacytoid DCs (pDCs) and the monocyte-derived DCs (moDCs, TipDCs) (125). In contrast to cDCs, pDCs are spherical in shape rather than possessing a dendritic morphology and express distinct surface markers B220, Siglec H, and Bst-2 in mice (128). pDCs are found in both lymphoid and non-lymphoid tissues and express both TLR7 and TLR9, which sense viral RNA and DNA, respectively. As a result, pDCs are important producers of type 1 interferon in response to viral infections (129). Type 1 interferon and IL-6 production by pDCs has also been shown to drive plasma cell differentiation from activated B cells (130). Monocyte-derived DCs are found in the periphery, where some also have the ability to migrate to lymphoid tissues upon acquiring antigen. Two types of monocytes have been characterized in mice (131). $CX_3CR1^{lo}CCR2^{hi}Ly6C^{hi}$ cells are known as inflammatory monocytes that home to sites of inflammation and can give rise not only to inflammatory macrophages but also inflammatory DCs (moDCs) and TNF and inducible nitric oxide synthase (iNOS)-producing DCs (TipDCs) (132-134). Patrolling monocytes ($CX_3CR1^{hi}CCR2^{lo}Ly6C^{lo}$), which initially contribute to early inflammation and then transition to tissue repair, can also differentiate into DCs (132). In addition, DCs can play a regulatory role in

controlling inflammation, but whether this depends on certain committed DC subsets and/or the local tissue conditions remains to be established (125).

Given the importance of DCs in the immune system, it comes as no surprise that they are essential in controlling *Toxoplasma* infection in vivo. In fact, although *Toxoplasma* can infect any nucleated cell, DCs serve as preferential targets. Transient ablation of all conventional $CD11c^+$ DCs via a diphtheria toxin approach resulted in decreased IL12 production and acute susceptibility to intraperitoneal *Toxoplasma* infection (135). In addition, DC IL12 production was found to be dependent on MyD88/TLR signaling *in vivo* when Cre recombinase was used to delete MyD88 exclusively in $CD11c^+$ cells (136). However, CD11c can also be expressed by other cell types including pDCs, macrophages, inflammatory monocytes and NK cells (137). A recent study identified a new marker unique to the conventional DC (cDC) lineage, the transcription factor zDC (Zbtb46, Btbd4). Diphtheria toxin-mediated depletion of zDC-expressing cells resulted in increased parasite tissue burden and decreased numbers of $CD4^+$ T cells, but not to the extent that that depletion of CD11c did, indicating that CD11c-expressing non-cDCs also play important roles in immunity to *Toxoplasma* (138). Indeed, recruitment of inflammatory monocytes to sites of infection has been shown to be crucial in controlling parasite replication during acute toxoplasmosis (139, 140). Although inflammatory monocytes and DCs derived from them are important in the local production of IL12 at infection sites (140, 141), lymphoid-resident $CD8^+$ DCs and the related $CD103^+$ peripheral DCs are the key systemic early IL12 producers during *in vivo* infection. CD8+ splenic DCs are also the primary IL12 producers in response to intravenous soluble tachyzoite antigen administration (142). pDCs (143), macrophages (144, 145), and neutrophils (146, 147) have also been shown to produce IL12 in

response to *Toxoplasma in vitro* but IL12 from these cell types is not likely to be critical *in vivo*. In addition to serving as key IL12 producers and initiators of adaptive immunity, DC also acquire a hypermotility phenotype upon *Toxoplasma* infection and subsequently potentiate parasite dissemination throughout the host (148-150).

Innate sensing of *Toxoplasma*

Early recognition of the parasite by cells of the innate immune system is crucial in shaping the ensuing immune response and outcome of infection. The Toll-like receptor (TLR) pathway dependent on the adaptor protein MyD88 has emerged as the major means of innate parasite recognition. Mice deficient in MyD88 are highly susceptible to *Toxoplasma* infection, with impaired production of the key Th1 cytokines IL12 and IFNγ (151, 152). Mice lacking MyD88 solely in $CD11c^+$ cells are also acutely susceptible, emphasizing the important role DCs play in initial pathogen sensing and immune initiation (136). Various parasite molecules have been identified as inducers of specific TLR signaling. The cell surface TLRs 2 and 4 can recognize parasite glycosylphosphatidylinositol (GPI) anchored proteins, but have little impact on survival of acute infection (153, 154). TLR11 was initially identified as the sensor of *Toxoplasma* profilin, a protein that plays an essential role during parasite invasion (155). Parasite profilin-induced TLR11 signaling results in IL12 production by DC; however, deletion of this TLR alone does not recapitulate the severity of the MyD88 deficient phenotype (155, 156). Recently, TLR12 has been recognized as the key sensor of parasite profilin, as mice deficient in this TLR demonstrate acute susceptibility to *Toxoplasma* (157). Furthermore, the nucleic acid-sensing (NAS) TLRs 7 and 9 can sense parasite RNA and DNA, respectively, although deficiency of either TLR has little to no impact on infection survival (158). It has recently become apparent,

however, that the parasite-sensing endosomal MyD88-coupled TLRs as a group (TLRs 7, 9, 11 & 12) are essential to mediate resistance to infection, as mutation of the chaperone UNC93B1 demonstrates (158). However, human cells lack TLRs 11 and 12 while TLRs 7 and 9 respond to parasite RNA and DNA with robust pro-inflammatory cytokine production. Therefore, the NAS-TLRs may play a more vital role during human toxoplasmosis (158).

Toxoplasma recognition can also be achieved through MyD88-independent pathways. Studies in infected bone marrow-derived macrophages have demonstrated MyD88-, G_iPCR-, and CCR5-independent IL12 production, with the amount dependent on parasite genotype and requiring p38 MAPK autophosphorylation (159, 160). Another parasite molecule, cyclophilin-18 (TgCyc18), has been shown to induce splenic DC IL12 production in dependence upon the chemokine receptor CCR5, a G_iPCR (161, 162). In addition, TgCyc18 and CCR5 have been shown to contribute to stage conversion to bradyzoites via NO production and to elicit IL12 and TNFα from macrophages (163). G_iPCRs other than CCR5 have also been implicated in *Toxoplasma* sensing, as the parasite triggers G_iPCR and PI3K-dependent phosphorylation of PKB/AKT in the absence of CCR5 (164). This pathway contributes to inhibition of apoptosis in *Toxoplasma*-infected cells. In addition, the parasite induces PI3K-dependent chemokine production in infected macrophages (165). Finally, MyD88-independent protection occurs *in* vivo as MyD88-deficient mice vaccinated with an attenuated uracil auxotroph strain survive challenge with a virulent strain (152). Thus, *Toxoplasma* initiates recognition in innate immune cells through a combination of MyD88-dependent and independent pathways, ultimately leading to development of a protective Th1 immune response.

Regulation of the immune response to *Toxoplasma*

Although induction of a strong Th1 immune response is normally protective, overproduction of pro-inflammatory cytokines can lead to tissue destruction and death. Therefore, the host must counteract the inflammation through production of key anti-inflammatory mediators including IL10, TGFβ, and IL27. Mice lacking IL10 are acutely susceptible to *Toxoplasma* infection by both the intraperitoneal and oral routes (166, 167). In these mice, pathogen replication is controlled, but overproduction of IL12, IFNγ, and TNFα leads to necrotic tissue death. The source of IL10 required to abrogate immunopathology is derived from IFNγ^{+}, T-bet^{+} Th1 cells that are negative for the T regulatory (Treg) cell marker Foxp3 (168). TGFβ also plays a role in immune regulation. When mice are depleted of TGFβ and infected with *Toxoplasma* via the oral route, they succumb to ileitis due to removal of a key immunoregulatory function of intraepithelial lymphocytes (169). The cytokine IL27 is also critical as demonstrated through infection of IL27 receptor knock-out mice. These mice develop lethal disease characterized by overproduction of IFNγ, enhanced T cell proliferation, and persistence of highly activated T cells (170). In addition, chronically infected mice develop severe neuroinflammation in association with the cytokine IL17 in the absence of the IL27R (171). IL27 also drives formation of a unique subset of Tregs responsible for control of immunopathology at mucosal sites during oral *Toxoplasma* infection (172). Thus, the host immune system has devised mechanisms of limiting parasite growth through a robust Th1 response while simultaneously limiting immunopathology.

PARASITE-MEDIATED EVASION OF INNATE IMMUNITY

In order for *Toxoplasma* to survive long enough to successfully establish chronic infection, the parasite must evade the destructive consequences of the Th1 response. In part, this is achieved at

the level of the infected cell through establishment of the non-fusogenic intracellular niche known as the parasitophorous vacuole. However, as described previously, the host cell possesses MyD88-dependent and independent means of recognizing the parasite, which are capable of inducing pro-inflammatory cytokine production and parasite destruction. Thus, the parasite must employ mechanisms to block immune cell recognition within infected cells. This is achieved primarily through interference with host cell signaling and transcriptional responses.

Inhibition of TLR signaling

Toxoplasma is known to block the expression of a wide variety of pro-inflammatory cytokines and chemokines normally induced by TLR signaling (173). In particular, the parasite inhibits lipopolysaccharide (LPS) signaling through TLR4, resulting in suppression of IL-12 and TNF-α in infected macrophages (174, 175). After ~12 hours, parasite infection leads to renewed IL-12 synthesis, but TNF-α remains inhibited (174). In addition, the parasite can block MyD88-independent TLR3 signaling by blocking IL-12 and IFN-β production after poly I:C stimulation (176). Further evidence of inhibition of TLR-induced cytokine production is found in other cell types, including bone marrow-derived dendritic cells (BMDCs) (177), splenic DCs (150), and peritoneal macrophages (175). Infected immature BMDCs also fail to upregulate MHCII and co-stimulatory molecules in response to LPS, with resultant deficits in T cell activation (177).

The blockade in TLR-triggered cytokine production is accompanied by interference with the main downstream effectors of TLR signaling, NF-κB and the MAP kinase cascade. This should come as no surprise as both pathways play important roles in the host immune response to the parasite (178, 179). *Toxoplasma* infected macrophages display a transient, early defect in NF-κB

nuclear accumulation (180-183). However, the early blockade in NF-κB nuclear accumulation may not be the only mechanism of suppression given that nuclear accumulation resumes after 6 hours of infection (180). The parasite is also able to impair chromatin remodeling at the TNF-α promoter in infected macrophages, preventing binding of NF-κB along with other transcription factors and thereby blocking transcription of TNF-α in response to LPS stimulation (184). *Toxoplasma* can also induce transient activation of MAP kinase cascades including p38, ERK1/2 and JNK/SAPK in a human monocytic cell line and in murine macrophages (182, 185). However, LPS-induced MAPK activation, particularly activation of p38, is reduced in parasite-infected murine macrophages, contributing in part to impaired cytokine production (182). The mechanism of how *Toxoplasma* inactivates TLR-induced MAPK signaling remains unclear. It is likely the parasite has evolved these mechanisms of inhibition of TLR signaling to prevent the host response to the parasite's own TLR ligands (176). Given that the oral route is the natural route of infection, it is also possible that blockade of TLR signaling is vital in preventing a hyper-inflammatory response secondary to gut flora exposure (186, 187).

Impact of secreted *Toxoplasma* virulence effectors

As previously described, *Toxoplasma* strains from North America and Europe comprise three main clonal lineages (I, II, and III) that differ in virulence in the mouse model (30). Forward genetic studies involving pair-wise crosses between strains of these three lineages have identified polymorphic parasite effectors that contribute to differences in acute virulence and impacts on host cell signaling. These effectors consist primarily of kinases or pseudokinases released from parasite rhoptries (ROP16, ROP18, ROP5) or dense granules (GRA15) upon or shortly after cell invasion (188).

ROP18 is an active parasite serine/threonine rhoptry kinase whose expression is high in type I and II strains but low in type III strains (189, 190). Transgenic expression of ROP18 from a type I or type II strain in a type III strain confers enhanced virulence in the latter, confirming ROP18's role in strain-specific virulence (189, 190). After parasite invasion, the host responds through recruitment of immunity-related GTPases to the PVM, under the influence of IFNγ (114, 115). However, IRG recruitment is impaired in type I strains (191). Upon invasion by a type I parasite, ROP18 is secreted and localizes to the surface of the parasitophorous vacuole membrane (PVM), where it catalyzes phosphorylation of several IRGs including IRGA6, IRGB6, and IRGB10 (192, 193). IRG phosphorylation results in their reduced recruitment to the PVM, preventing IRG-mediated destruction of the parasite and helping explain the high virulence of type I strains (192, 193). However, these results do not explain why type II strains are unable to block IRG recruitment and subsequent parasite destruction, given their high level of ROP18 expression. In addition, although type I parasites deficient in ROP18 are highly avirulent *in vitro*, virulence is only partially diminished *in vivo* (192). In addition to impairing innate immunity through effects on IRG recruitment, ROP18 has been shown to phosphorylate the ER-resident activating transcription factor 6 beta (ATF6β) (194), resulting in impaired initiation of $CD8^+$ T cell-mediated adaptive immunity by DCs (195).

Further knowledge of ROP18 function arose when ROP5 was identified as another key virulence determinant. Type I parasites lacking ROP5 were found to be highly attenuated *in vivo*, by over one million-fold (196, 197). However, ROP5 is a pseudokinase, and therefore does not require kinase activity to mediate its function (198). Further work identified interactions between ROP5 and ROP18. ROP5 has been shown to impair oligomerization of IRGA6 (199, 200), while also

allosterically activating ROP18, thereby enhancing ROP18 phosphorylation of IRGA6 and IRGB6 (199, 201). The ROP5 variant present in type II strains is considered inactive, explaining why high levels of ROP18 expression is not enough to confer the virulence of type I strains. By contrast, ROP18 from type II strains can enhance virulence when combined with ROP5 of type I and III strains (189, 190). Although type III strains express functional ROP5, the absence of sufficient ROP18 explains the avirulence of this strain. ROP5 likely plays a role in the activation of other parasite kinases, although additional targets have yet to be identified (201).

The rhoptry kinase ROP16 is another key virulence determinant with activity in type I and III strains, but not type II strains (202). This kinase, although predicted to be a serine/threonine kinase, has been shown to directly phosphorylate host STAT3 (203) and STAT6 (204) transcription factors on key tyrosine residues. ROP16 from a type I or III parasite is required for maintenance of STAT3/6 phosphorylation in infected macrophages (202, 205). A key amino acid substitution at position 503 accounts for the difference in activity of type I/III ROP16 versus that of type II (203). Prolonged ROP16-dependent STAT3 activation leads to reduced IL12 production during infection with type I or III strains, and also impairs LPS-induced synthesis of IL12 and TNFα (202, 205). Despite these impacts on host gene transcription, ROP16 appears to have little impact on acute virulence following intra-peritoneal infection, as deletion of ROP16 does not reduce mouse mortality (205). However, parasite growth and dissemination were enhanced *in vivo* (205). This effect may be the result of ROP16 impacts on nutrient availability for the parasite. ROP16 also targets STAT6, leading to increased host arginase I expression, an enzyme that degrades the amino acid arginine (205). The impact of increased arginase activity on the parasite is complex, as both arginine and the polyamine products of arginine degradation

are essential for *Toxoplasma* growth; however, arginine appears to be limiting *in vivo* (206). STAT6 activation by type I and III parasites also contributes to early polarization of infected macrophages to an alternative activation (M2) phenotype, promoting an anti-inflammatory state (207). Finally, ROP16 has also recently been shown to cause sustained phosphorylation and nuclear translocation of STAT5 in infected cells, although what role this may play remains to be determined (208).

Type II strains produce more IL12 than Type I or III strains in infected macrophages (145, 159). In part this is due to the lack of ROP16-dependent STAT3 activation that limits MyD88-dependent IL-12 production by type I and III strains. However, type II strains also express GRA15, a dense granule protein that induces substantial NF-κB activation and subsequent IL12 production (209). GRA15 activates NFκB in a TLR-independent fashion, but the exact mechanism is unclear (209). In contrast to type I ROP16, type II GRA15 induces classical activation of macrophages, promoting antimicrobial activity (207). A recent study found that type I ROP16 and type II GRA15 must act synergistically to promote host resistance to *Toxoplasma*-induced ileitis during oral infection, limiting both parasite burden and inflammation (208). The influence of type I ROP16 in that study occurred independently of STAT3/6, suggesting that ROP16-activated STAT5 may instead be important during oral infection, but this awaits formal proof (208). Regardless, it is clear that parasite-mediated manipulation of the innate immune response requires a carefully orchestrated interplay between parasite proteins and the host molecules they target, as summarized in Figure 1.4.

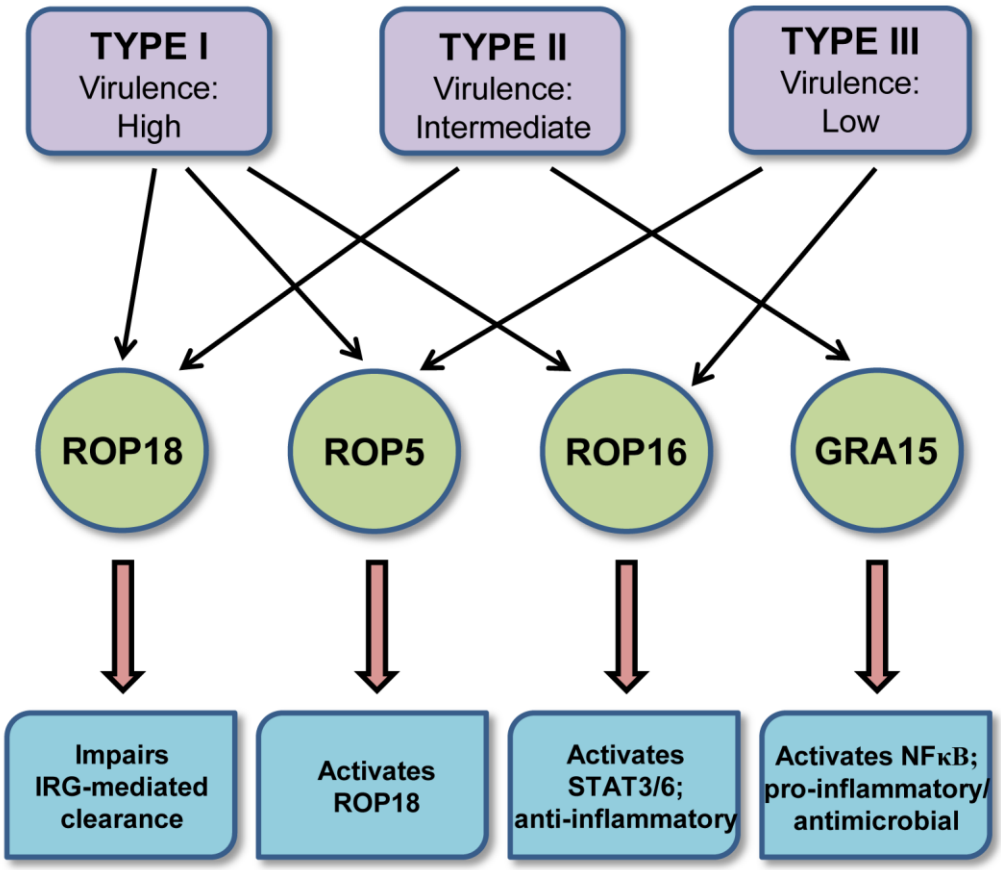
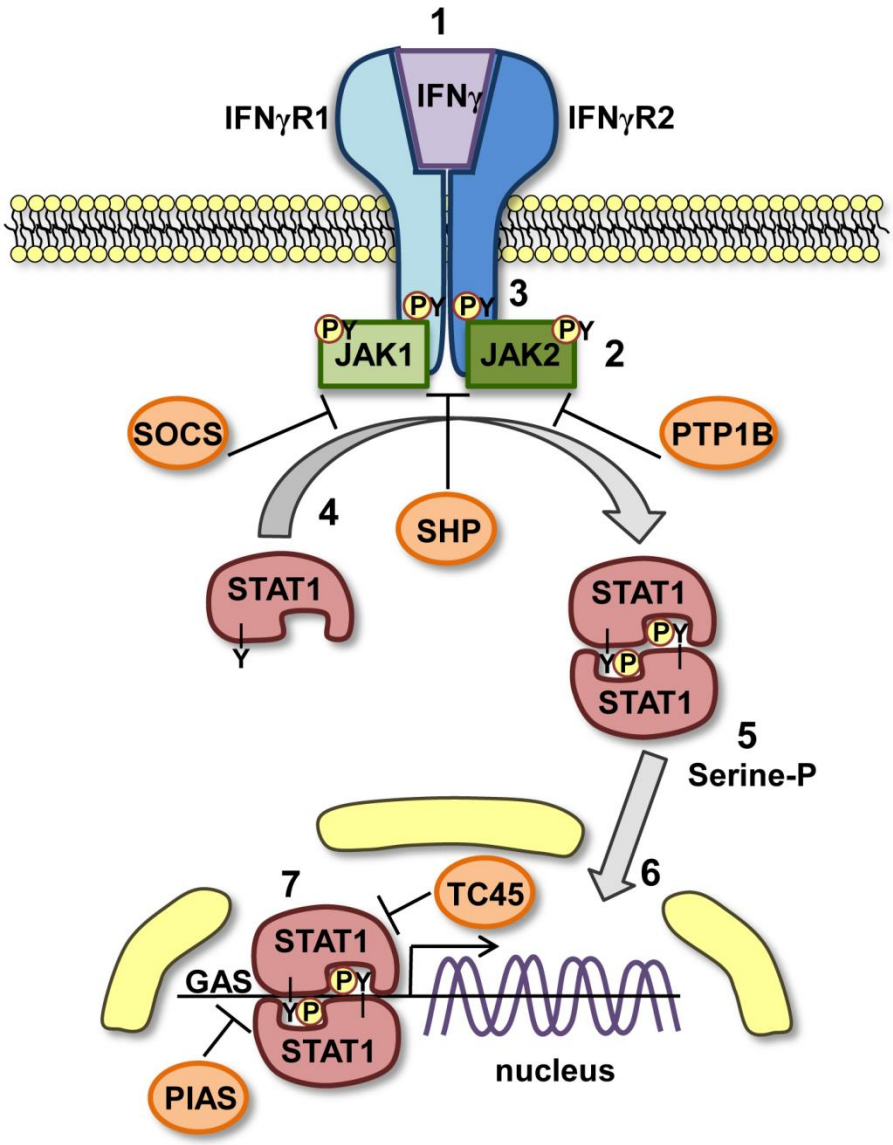


Figure 1.4. The relationships between *Toxoplasma* strain types, known virulence effectors, and impacts on host cell immune responses. Type I strains possess active ROP18, ROP5, and express high levels of ROP16. Type II strains possess active ROP18 and GRA15. Type III strains possess active ROP5 and express high levels of ROP16. Impacts of ROP18, ROP5, ROP16, and GRA15 on the infected host cell's immune response are indicated in the blue boxes.

Interference with IFNγ/STAT1 activity

Given that IFNγ is considered the chief mediator of immunity, it is no surprise that *Toxoplasma* also targets this cytokine and its signaling pathway as part of an immune evasion strategy. To better appreciate how the parasite targets this pathway, it is first necessary to review how IFNγ signals. IFNγ exerts many of its effects on antimicrobial function through a Janus kinase (JAK)/ signal transducer and activator of transcription 1 (STAT1)-dependent pathway. In the canonical pathway (as reviewed in (210) and summarized in Figure 1.5), IFNγ binds to its receptor subunits (IFNγR1 and IFNγR2), causing them to oligomerize. This conformational change results in auto-phosphorylation and activation of JAK2, which in turn activates JAK1 via trans-phosphorylation. JAK1 phosphorylates tyrosine residue 440 within the intracellular domains of the IFNγR1 subunits, which in turn recruits STAT1 monomers that bind via SH2 domains. STAT1 is then phosphorylated by JAK1 on a key tyrosine residue, Y701. Activated STAT1 monomers dissociate from the receptor complex and form STAT1 homodimers through reciprocal SH2-phosphotyrosine interactions, with subsequent translocation to the nucleus. There, they recognize interferon gamma-activated sequence (GAS) elements ($TTCN_3GAA$) in the promoters of responsive genes and initiate transcription (211). Phosphorylation of a key serine residue, S727, is required to achieve maximal transcriptional activity, but not for DNA binding (212-214). A variety of kinases have been identified as contributors to STAT1 serine phosphorylation, depending on the type of cell and stimulus (215). Calcium/calmodulin-dependent protein kinase II/CaMKII (216) and PI3K/AKT/PKCδ-dependent pathways (217, 218) have been implicated in IFNγ-triggered serine phosphorylation, while p38 MAPK involvement has also been demonstrated with stress stimuli (219).

Figure 1.5. The canonical JAK/STAT1 signaling pathway initiated by IFNγ. IFNγ binds to both receptor subunits, IFNγR1 and IFNγR2, causing them to oligomerize (1). This results in autophosphorylation and activation of JAK2, which in turn phosphorylates and activates JAK1 (2). The activated JAKS in turn phosphorylate tyrosine residues in cytoplasmic portions of the receptor subunits (3). STAT1 is recruited to the receptor, interacting with the phospho-tyrosine residues of the receptor through an SH2 domain. JAK1 then phosphorylates STAT1 on tyrosine residue 701, causing STAT1 to dissociate from the receptor and dimerize (4). At some point, phospho-STAT1 dimers are also phosphorylated on serine residue 727, which is required for maximal transcriptional activity (5). Phospho-STAT1 dimers translocate to the nucleus (6), where they recognize interferon gamma-activated sequence (GAS) elements in the promoters of responsive genes, initiating transcription (7). The IFNγ/STAT1 pathway also undergoes negative regulation, as indicated by the orange ovals. Suppressor of cytokine signaling (SOCS)-1, protein tyrosine phosphatase 1B (PTP1B), and Src homology 2 domain-containing phosphatases (SHP) 1 and 2 target the JAKs and IFNγ receptor subunits for inactivation. T cell phosphatase 45 (TC45) serves as the primary phosphatase for STAT1 inactivation in the nucleus. PIAS1 interferes with STAT1 DNA binding while PIASy acts as a co-repressor of STAT1.


1
IFNγ
IFNγR1
IFNγR2
PY
PY
3
PY
JAK1
JAK2
PY
2
SOCS
PTP1B
4
SHP
STAT1
Y
STAT1
PY
YP
STAT1
5
Serine-P
6
7
TC45
STAT1
GAS
PY
YP
STAT1
PIAS
nucleus

However, JAK/STAT signaling is not quite that simple, as various studies have demonstrated (reviewed by Mohr et al (220)). In some cases, pre-formed dimers of the cytokine receptors are present, which are then stabilized by ligand binding rather than ligand binding triggering dimerization/oligomerization. Pre-formed dimers of STATs may also exist, undergoing a conformational change to achieve activation upon phosphorylation. In addition, a fraction of unphosphorylated STAT molecules can be found in the nucleus in the steady-state as a result of constant nucleo-cytoplasmic shuttling (220). These U-STATs can also mediate gene regulation (221). STATs may also play additional roles outside of the nucleus under certain conditions. Finally, STATs such as STAT1 undergo additional post-translational modifications in addition to tyrosine and serine phosphorylation that affect STAT activity, including methylation, SUMOylation, and ISGylation (ISG15 – a small ubiquitin-like protein) (210).

Depending on the specific target gene involved, STAT1 cooperates with other transcription factors to facilitate recruitment of histone modifying enzymes necessary for establishment of permissive chromatin at gene promoters. In turn, chromatin binding and initiation of transcription by RNA polymerase II occurs. Several different covalent histone modifications can occur including methylation, acetylation, phosphorylation, ubiqutinylation, and SUMOylation, which can impact transcription positively or negatively (222). Histone acetylation at key residues is more frequently associated with permissive chromatin. Serine phosphorylation at S727 is important in the recruitment of the key histone acetyltransferase, CBP/p300, to STAT1-regulated promoters (223). In addition, a unique histone methyltransferase, DOT1L, is required for STAT1 activation of *Irf1* expression (224). However, chromatin remodeling complexes such as the BAF (BRG/BRM) complex also play a role by disrupting histone/DNA contacts in an

ATP-dependent manner. BAF has also been implicated in the remodeling of some STAT1-responsive genes, such as the class II transactivator (CIITA) (225) although not for *Irf1* (226). In addition, STAT1 can interact with other transcription co-factors or DNA binding proteins including Nmi, Sp1, Mcm5 and Mcm3 (210). Thus, the activation of transcription by STAT1 is complex, involving cooperation with wide variety of other transcription factors and histone modifiers that differ depending on the gene and cell type.

Finally, STAT1 activity in response to IFNγ stimulation must be tightly regulated to control biologic functions (Figure 1.5). Phosphorylated STAT1 in the nucleus is dephosphorylated by a key nuclear phosphatase, the nuclear isoform of T cell phosphatase 45 (TC45), allowing return of unphosphorylated STAT1 to the cytoplasm (227). Another form of regulation in nucleus involves interaction of STAT1 with the protein inhibitor of activated STAT (PIAS) family of proteins. PIAS1 binds phosphorylated STAT1 and blocks its recruitment to promoters; however, PIAS1 expression is low in DCs and only regulates a subset of STAT1-dependent genes which do not include *Irf1* (228). PIASy also regulates STAT1 and is found in DCs; however it functions as a co-repressor of STAT1 without affecting DNA binding (229). Un- or partially de-phosphorylated STAT1 can also be conjugated to small ubiquitin-like modifier (SUMO) at residue K703, which prevents hyperresponsiveness to IFNγ (230, 231). SUMOylation in turn increases STAT1 solubility, preventing formation of nuclear paracrystalline arrays that would normally protect other phosphorylated STAT1 dimers from dephosphorylation (230, 231). It is also necessary to down-regulate receptor/JAK-mediated STAT1 phosphorylation in the cytoplasm, which can be accomplished by various phosphatases. These phosphatases target JAKs or cytokine receptors and include the protein tyrosine phosphatase 1B and the SH2-

containing phosphatases SHP1 and SHP2 in the case of STAT1 regulation (210). In addition, the suppressor of cytokine signaling (SOCS) family of proteins participates in regulation by direct inhibition of receptor/JAK signaling or indirectly by targeting signaling components for ubiquitin/proteasome-mediated degradation (210). SOCS1 is the key SOCS protein known to regulate IFNγ responses (232, 233). Finally, STAT1 can also be expressed as an alternative splice variant, STAT1β, which lacks a portion of the C-terminal transactivation domain and therefore acts as a dominant negative form of STAT1 (210).

An understanding of the complexities of IFNγ-mediated STAT1 activation, activity and regulation sets the stage for determining how *Toxoplasma* may target this pathway for immune evasion. STAT1 is essential for survival of acute *Toxoplasma* infection *in vivo* as it is required for key IFNγ-triggered antimicrobial effector mechanisms (234, 235). The parasite has been shown to potently repress expression of certain IFNγ-inducible genes including those involved in antigen presentation (MHCII, MHCI, CIITA) (236-240), the key transcription factor IRF1 (238, 240-242), the chemokine monokine induced by IFNγ (MIG) (243), and various antimicrobial effector molecules including iNOS/NO (234, 243-246) and the p47 family of GTPases (234, 243). These effects have been demonstrated in a variety of cell types including primary bone marrow-derived macrophages, the RAW264.7 mouse macrophage cell line, human fibroblasts, peritoneal macrophages, astrocytes, and microglia. In addition, a global picture of *Toxoplasma*-inhibition of IFNγ transcription has been achieved in human fibroblasts (241), murine macrophages (247), and the RAW264.7 cell line (242) through microarray studies.

It has become clear that targeting the IFNγ pathway to avoid immune-mediated elimination is a key defensive strategy for the parasite. A question remains as to how the parasite achieves this. One study in the RAW264.7 cell line showed that the parasite can induce expression of *Socs1*, thereby decreasing IFNγ-induced STAT1 phosphorylation and preventing STAT1 activity (243). Another study suggested that the parasite may enhance dephosphorylation of STAT1 within the nucleus of infected human fibroblasts (241). However, most studies confirm that *Toxoplasma* does not interfere with IFNγ-induced STAT1 phosphorylation and nuclear translocation (240, 242, 247). The mechanism appears to involve inhibition of transcription in the nucleus, with evidence of impairment of chromatin remodeling at certain IFNγ-regulated gene promoters (247). How exactly *Toxoplasma* orchestrates this potent inhibition of IFNγ/STAT1-mediated transcriptional activity remains a question for further study.

OUTLINE OF DISSERTATION RESEARCH

IFNγ and its key signaling intermediate, STAT1, are crucial to host defense during *Toxoplasma* infection. The parasite possesses many mechanisms for immune evasion, but paramount among them is evasion of the toxoplasmacidal IFNγ response. Clues as to how the parasite carries out this subterfuge have emerged in macrophages, fibroblasts, astrocytes/microglia, and various cell lines. However, the precise mechanism by which *Toxoplasma* hijacks this pathway remains unclear. In addition, the importance of this evasion in the dendritic cell has not been examined, despite its essential role in the immune response to the parasite.

This dissertation examines the impact of *Toxoplasma* on the IFNγ/STAT1 pathway in murine dendritic cells. **Chapter 2** reveals an unexpected finding that the parasite can trigger sustained

STAT1 phosphorylation and nuclear translocation in dendritic cells in the absence of exogenous IFNγ. Furthermore, IFNγ stimulation of cells pre-infected with the parasite results in synergistic levels of phosphorylated STAT1 as well as binding of an aberrant STAT1-containing complex to consensus nucleotides. Despite evidence of activation, STAT1 cannot bind to native promoters and thus transcription of IFNγ/STAT1-responsive genes remains blocked. The importance of parasite-mediated blockade of the IFNγ/STAT1 pathway in dendritic cells is demonstrated. **Chapter 3** further explores the mechanisms behind inhibition of IFNγ/STAT1-responsive gene transcription, parasite-triggered STAT1 phosphorylation, and synergy with IFNγ. Inhibition of gene transcription is shown to occur independently of parasite strain type and the virulence factor ROP16. Key secreted parasite molecules are eliminated as initiators of STAT1 phosphorylation. In addition, signaling pathways known to be triggered by *Toxoplasma* are excluded as causes for STAT1 phosphorylation and synergy with IFNγ. Finally, downregulation of IFNγ-induced *Socs1* expression by the parasite is shown, which explains the synergistic phosphorylated STAT1 levels observed. **Chapter 4** summarizes the data and results from chapters 2 and 3 and discusses the overall relevance and importance of these findings to the fields of *Toxoplasma* biology and immune evasion of IFNγ-mediated responses by pathogens. Unanswered questions and future directions for this project are also discussed.

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CHAPTER 2

Toxoplasma gondii Triggers Phosphorylation and Nuclear Translocation of Dendritic Cell STAT1 While Simultaneously Blocking IFNγ-Induced STAT1 Transcriptional Activity[1]

[1] Reprinted from Anne G. Schneider, Delbert S. Abi Abdallah, Barbara A. Butcher and Eric Y. Denkers. *Toxoplasma gondii* triggers phosphorylation and nuclear translocation of dendritic cell STAT1 while simultaneously blocking IFNγ-induced STAT1 transcriptional activity. (2013) *PLoS One* 8: e60215.

Abstract

The protozoan *Toxoplasma gondii* actively modulates cytokine-induced JAK/STAT signaling pathways to facilitate survival within the host, including blocking IFNγ-mediated STAT1-dependent proinflammatory gene expression. We sought to further characterize inhibition of STAT1 signaling in infected murine dendritic cells (DC) because this cell type has not previously been examined, yet is known to serve as an early target of in vivo infection. Unexpectedly, we discovered that *T. gondii* infection alone induced sustained STAT1 phosphorylation and nuclear translocation in DC in a parasite strain-independent manner. Maintenance of STAT1 phosphorylation required active invasion, but intracellular parasite replication was dispensable. The parasite rhoptry protein ROP16, recently shown to mediate STAT3 and STAT6 phosphorylation, was not required for STAT1 phosphorylation. In combination with IFNγ, *T. gondii* induced synergistic STAT1 phosphorylation and binding of aberrant STAT1-containing complexes to IFNγ consensus sequence oligonucleotides. Despite these findings, parasite infection blocked STAT1 binding to the native promoters of the IFNγ-inducible genes *Irf-1* and *Lrg47*, along with subsequent gene expression. These results reinforce the importance of parasite-mediated blockade of IFNγ responses in dendritic cells, while simultaneously showing that *T. gondii* alone induces STAT1 phosphorylation.

Introduction

Toxoplasma gondii is among the most successful parasitic microorganisms, infecting virtually all warm-blooded animals. Up to one-third of the human population worldwide is infected with this protozoan (1). Most infections are asymptomatic or present with mild flu-like symptoms, and the parasite establishes life-long infection characterized by presence of latent cysts in host tissues. Should immunocompromise develop, as in organ transplant recipients and AIDs patients, cyst reactivation can occur and lead to deadly encephalitis (2). Fetuses can also become infected *in utero* during primary maternal infection with devastating consequences including blindness, mental retardation, and death (3). Three main clonal lineages of the parasite have been identified in humans and domestic animals in North America, which differ in terms of virulence in mice (4). Type I strains are the most virulent (LD50=1), resulting in death of the mouse during acute infection, whereas types II and III strains are much less virulent and capable of establishing chronic infection (5). All three strain types can cause disease in humans, although type I strains may be more common in cases of ocular disease (6). Recently, a fourth clonal lineage has been identified in wildlife populations of North America (7, 8).

Cell-mediated immunity is crucial to limiting severity of *Toxoplasma* infection. Widely considered the major mediator of host resistance is the cytokine interferon-gamma (IFNγ), produced primarily by T cells and NK cells. This cytokine is necessary for the control of intracellular parasite replication both *in vivo* and *in vitro*, in both mice and humans (9-14). Among many effects, IFNγ activates antimicrobial effector mechanisms such as the immunity-related p47-GTPases that facilitate degradation of the parasitophorous vacuole (15-17).

Critical to the action of IFNγ in inducing antimicrobial effector mechanisms is the transcription factor signal transducer and activator of transcription 1 (STAT1) (18, 19). Indeed, in the absence of STAT1, *Toxoplasma*-infected mice succumb rapidly to infection (20, 21). In the classical signaling pathway, IFNγ binds its receptor and signals through a Janus kinase (JAK)/STAT1 cascade. Activated JAKs phosphorylate the IFNγ-receptor, permitting recruitment of STAT1. STAT1 is phosphorylated on a key tyrosine residue, Y701, and translocates as a dimer to the nucleus to initiate transcription, binding to gamma-activated sequence (GAS) elements in the promoters of responsive genes (22-24). Phosphorylation at a key serine residue, S727, is thought to facilitate maximal STAT1 transcriptional activity (25-27). One of the key early response genes regulated by STAT1 is interferon regulatory factor-1 (*Irf1*) (28, 29), itself a transcription factor, the absence of which also increases susceptibility to *Toxoplasma* infection (30).

Given the vital impact on parasite survival, it may not be surprising that *Toxoplasma* possesses mechanisms to counteract the IFNγ/STAT1 pathway. This has been demonstrated in a variety of cell types, including bone marrow-derived macrophages (BMDM), microglia, astroglia, the monocyte/macrophage RAW264.7 cell line, as well as human and murine fibroblasts. The parasite has been shown to block IFNγ-mediated upregulation of MHC class I and II molecules, class II transactivator (CIITA), inducible nitric oxide synthase, the chemokine monokine induced by IFNγ (MIG), interferon-inducible GTPase 1 (IIGP1) and IRF1 (31-39). In addition, genome-wide microarray analyses in human fibroblasts and murine macrophages have described global inhibition of IFNγ-mediated gene expression in infected cells (40-42).

The molecular mechanism of inhibition remains unclear. Some discrepancy exists as to whether *Toxoplasma* targets STAT1 itself. One study performed in RAW264.7 cells at a high multiplicity of infection (MOI) concluded that infection blocked IFNγ-mediated STAT1 phosphorylation, likely via upregulation of suppressor of cytokine signaling-1 (SOCS1) (39), while another study implicated partial STAT1 dephosphorylation in the nuclei of infected human fibroblasts (40). However, other studies found that STAT1 phosphorylation and nuclear translocation were unimpaired in infected cells, suggesting instead a block in IFNγ-mediated gene transcription (38, 41, 42). The mechanism by which this occurs likely involves impaired recruitment of histone modifying enzymes such as BRG-1 to certain gene promoters, thereby rendering native chromatin inactive for transcription (41). However, the particular parasite factors or signaling pathways involved in the inhibition remain unknown.

In our study, we sought to investigate the impact of *Toxoplasma* infection on the IFNγ/STAT1 pathway in primary bone marrow-derived murine dendritic cells (BMDC). Dendritic cells serve as an important early target of in vivo infection, playing a critical role in parasite dissemination throughout the host (43-45). They also play a pivotal role in immune initiation (46), and their genetic deletion results in acute susceptibility to *T. gondii* (47, 48). We unexpectedly discovered that *Toxoplasma* alone induces STAT1 phosphorylation and nuclear translocation in infected BMDC, regardless of strain type. In addition, we observed synergistic STAT1 phosphorylation when infected cells were treated with IFNγ. Electrophoretic mobility shift assays (EMSAs) revealed the presence of an aberrant GAS-binding complex that increased during the infection period in the presence of IFNγ. However, transcription of IFNγ/STAT1-responsive genes was impaired in infected cells. Binding of STAT1 to the native *Irf-1* promoter in response to IFNγ

was also abrogated. These findings reveal that *Toxoplasma* triggers phosphorylation of STAT1 that nonetheless is unable to act as a transcription factor for typical IFN-γ-regulated genes.

Materials and Methods

Ethics Statement

All experiments with animals in this study were performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocols were approved by the Institutional Animal Care and Use Committee at Cornell University (Permit Number: 1995-0057). All efforts were made to minimize animal suffering.

Mice and Parasites

Female C57BL/6 mice of 6-8 weeks of age were purchased from either the Jackson Laboratory or Taconic Farms. All mice were housed under specific pathogen-free conditions at the Transgenic Mouse Core Facility at Cornell University's College of Veterinary Medicine, which is accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care. Tachyzoites of the *Toxoplasma* strains RH (type I), PTG (type II), M774.1 (type III), *cps1-1* (type I attenuated strain), and ΔROP16 (RH/type I background) were maintained by biweekly passage on human foreskin fibroblast monolayers (American Type Tissue Collection) in DMEM (Life Technologies) supplemented with 1% bovine growth serum (Hyclone), 100 U/ml penicillin (Life Technologies), and 0.1 mg/ml streptomycin (Life Technologies). The RH parasites deficient in the rhoptry kinase ROP16 (ΔROP16) were generated as described previously (49) and were kindly provided by D. Bzik and B. Fox (Dartmouth Medical Center).

The attenuated uracil auxotroph strain *cps1-1* (50) was supplemented with 250 nM uracil during passage to permit replication. Parasite cultures were tested every 6-8 weeks for *Mycoplasma* contamination using a commercial PCR-ELISA based kit (Roche Applied Systems).

Bone Marrow-Derived Dendritic Cell (BMDC) Culture

Bone marrow was flushed from the femur and tibia of a C57BL/6 mouse and prepared as a single cell suspension in BMDC medium composed of RPMI 1640 (Fisher Scientific) supplemented with 10% fetal calf serum (Hyclone), 100 U/ml penicillin (Life Technologies), 0.1 mg/ml streptomycin (Life Technologies), 50 μM 2-mercaptoethanol (Sigma) and 20 ng/ml GM-CSF (Peprotech). Cells were plated on 100 x 15 mm standard sterile polystyrene Petri dishes (Fisher Scientific) and cultured for 9 days at 37°C in 5% CO_2. Fresh BMDC medium was added on day 3. On day 6, fresh BMDC medium containing 50 mM 2-mercaptoethanol was added. On day 8, 200 ng of GM-CSF was added per plate. On day 9, non-adherent cells (BMDC) were collected and resuspended in DMEM (Life Technologies) supplemented with 10% bovine growth serum (Hyclone), 50 mM 2-mercaptoethanol (Sigma) and the following reagents from Life Technologies: 100 U/mL penicillin (Life Technologies), 0.1 mg/ml streptomycin (Life Technologies), 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, and 3% HEPES (1M).

***In vitro* Infections and Stimuli**

Infection of BMDCs was accomplished through addition of tachyzoites to cell cultures at a ratio of 3:1 (parasites:BMDCs) followed by brief centrifugation (200 x g, 3 min) to initiate contact between cells and parasites. In other experiments, cells were treated with recombinant murine IFNγ (100 ng/mL, Peprotech) or first pre-infected with tachyzoites for two hours followed by

IFNγ treatment. For cytochalasin D experiments, BMDC were pretreated for 10 min at 4°C with cytochalasin D (Calbiochem) at a final concentration of 1 μM or with the solvent DMSO (Sigma) alone. Cells were then infected with tachyzoites or treated with recombinant murine IFNγ for 6 hours in the continued presence of the drug. For the supernatant transfer experiments, supernatants from cells infected with tachyzoites or treated with IFNγ overnight were collected, centrifuged to remove cellular debris, and filtered through 0.2 μM filters (Corning) prior to addition to untreated cells.

Immunoblot Analysis

The following primary antibodies from Cell Signaling were used in immunoblotting studies: anti-phospho-STAT1-Tyr 701 (catalog no. 9167), anti-phospho-STAT1-Ser727 (catalog no. 9177), and anti-PARP (catalog no. 9542). The Rab5a antibody (catalog no. sc-309) was obtained from Santa Cruz Biotechnology. Cells ($3x10^6$/sample) were fractionated into cytoplasmic and nuclear fractions using a nuclear extract kit (Active Motif) as directed. Samples were subsequently diluted with 2x reducing SDS sample buffer. After 5 min at 100°C, samples were separated by 10% SDS-PAGE and proteins were subsequently electrotransferred onto nitrocellulose membranes (Whatman). The membranes were blocked for one hour at room temperature in Tris-buffered saline containing 0.1% Tween 20, pH 7.6, (TBST) and 5% nonfat dry milk prior to the addition of primary antibody overnight at 4°C in TBST containing 5% bovine serum albumin (BSA) (Calbiochem). Membranes were subsequently washed with TBST prior to detection of primary antibody binding with a horseradish peroxidase-conjugated anti-rabbit IgG (Jackson ImmunoResearch, catalog no. 111-035-144) in TBST containing 5% nonfat

dry milk for one hour at room temperature. Membranes were washed in TBST prior to visualization of bands using a chemiluminescence detection system (Thermo Scientific).

Flow Cytometry

BMDC were infected with the type 1 parasite strain RH at a ratio of 0.5 parasites per cell and harvested for flow cytometric analysis after 20 hours. Samples were fixed for 10 minutes at room temperature with 3% formaldehyde, followed by permeabilization for 30 minutes at 4°C with ice-cold 100% methanol. Cells were plated at $2x10^6$ per well in a 96-well plate and washed twice with FACS buffer (1% BSA in PBS). Cells were stained with anti-phospho-STAT1-Tyr701 (Cell Signaling, catalog no. 9167) diluted in FACS buffer for 1 hour at room temperature. Samples were washed twice with FACS buffer prior to addition of an antibody mixture for 30 minutes at room temperature containing goat-anti-rabbit-Alexa Fluor 647 (Life Technologies, catalog no. A21245) to detect the primary p-STAT1 antibody, and anti-p30-FITC (Argene, catalog no. 12-132) to detect *Toxoplasma*-infected cells. Samples were again washed with FACS buffer prior to analysis on a FACS Calibur flow cytometer (BD Biosciences). Data were subsequently analyzed using FlowJo software (Tree Star).

Transcription Factor DNA-binding ELISA

The presence of activated STAT1 complexes capable of binding to consensus oligonucleotides was assessed in BMDC nuclear extracts using the TransAM STAT Family Transcription Factor Assay Kit (Active Motif), per manufacturer's instructions. BMDC were either infected with the RH strain of parasites (ratio of 3 parasites per cell) or treated with recombinant murine IFNγ

(Peprotech) for six hours prior to analysis. The STAT consensus nucleotide coated on the 96-well plates consisted of the following sequence: 5'-TTCCCGGAA-3'.

Electrophoretic Mobility Shift Assay (EMSA)

To further confirm STAT1 binding-activity in nuclear extracts, EMSA was performed using a LightShift Chemiluminescent EMSA kit, per manufacturer's instructions (Thermo Scientific). Briefly, complementary oligonucleotides (5'-CATTTCGGGGAAATCGATC-3' and 5'-GATCGATTTCCCCGAAATG-3'; IDT Technologies) designed against the GAS sequence of the *Irf-1* promoter (Ng et al, 2011) were biotinylated at the 5' end prior to annealing equimolar quantities of each to create a labeled probe. Equal volumes of nuclear extract (from $3x10^6$ cells) were then incubated for 20 minutes with 200 fmol of biotinylated probe, 1 μg poly(I)-poly(C), binding buffer (Thermo Scientific), 2.5% glycerol, 50 mM KCl, 1 mM DTT, and 1 mM EDTA. Supershift assays were performed by the addition of 2 μg of supershift grade rabbit anti-STAT1α p91 (Santa Cruz Biotechnology, sc-591x) or normal rabbit IgG (Santa Cruz Biotechnology, sc-2027) for 20 minutes on ice prior to the addition of nuclear extract at room temperature for an additional 20 minutes. The protein-DNA complexes were resolved on a native 5% polyacrylamide gel (Bio-Rad) in 0.5x TBE buffer prior to transfer to a positively charged nylon membrane (Bio-Rad). Transferred DNA was crosslinked to the membrane using a commercial UV light crosslinking instrument (UV Stratalinker 2400, Stratagene) at 120 mJ/cm^2 using the auto crosslink function. Shifts of the biotinylated DNA probe were detected using a streptavidin-horseradish peroxidase conjugate and chemiluminescent substrate (Thermo Scientific).

Quantitative Reverse Transcriptase PCR

BMDC ($3x10^6$) were treated with IFNγ and/or infected with the RH parasite strain prior to isolation of total RNA using the E.Z.N.A Total RNA Miniprep kit (Omega Bio-Tek). Samples were treated on column with DNase I (Agilent Technologies) during the total RNA isolation. cDNA was synthesized from total RNA using qScript cDNA SuperMix (Quanta Biosciences). Quantitative PCR was performed on cDNA samples using the SYBR green method (Quanta Biosciences) and the ABI Prism 7500 sequence detection system (Applied Biosystems). Expression of target genes was normalized to the housekeeping gene GAPDH, and the relative expression of treated samples versus an untreated control sample was calculated using the $\Delta\Delta C_T$ method. The primer sequences employed were: *Irf1* forward: 5'-TTGGCATCATGGTGGCTGT-3'; *Irf1* reverse: 5'-AAGGAGGATGGTCCCCTGTTT-3'; *Lrg47* forward: 5'-GAGACTGTGGCAACATTGTCCC-3', *Lrg47* reverse: 5'-CCGATGACTCGAAGTGCATTG-3'; *GAPDH* forward: 5'-AATGGTGAAGGTCGGTGTG-3', *GAPDH* reverse: 5'-GTGGAGTCATACTGGAACATGTA-3'; *Irf1* promoter forward: 5'-AGCTCTACAACAGCCTGATTTCCC-3', *Irf1* promoter reverse: 5'-GCGCCGCGAAGAAATCTAAACACT-3'.

Chromatin Immunoprecipitation (ChIP)

An anti-total-STAT1α antibody (ChIP grade, Santa Cruz Biotechnology, sc-591x) was used to precipitate STAT1-bound chromatin fragments. Normal rabbit IgG (Santa Cruz Biotechnology, sc-2027) was used as a negative control antibody. Assays were performed using the Magna ChIP G Chromatin Immunoprecipitation kit (Millipore, catalog no. 17-611) according to the manufacturer's instructions. Briefly, BMDC (10^7/sample) were collected and fixed in 1%

formaldehyde for 5 minutes at room temperature. Fixation was stopped by addition of glycine to the mixture. Samples were washed with cold PBS and resuspended in cell lysis buffer (Millipore) supplemented with protease inhibitors for 15 minutes at 4°C. Resulting nuclear pellets were spun down and resuspended in nuclear lysis buffer (Millipore) prior to chromatin shearing by sonication in an ice bath with twelve, thirty second pulses at high power using the Bioruptor (Diagenode). Immunoprecipitation of the sheared chromatin was carried out overnight at 4°C via addition of dilution buffer (Millipore), antibody (2 μg), and protein G magnetic beads. Beads were washed, protein/DNA crosslinks were reversed, proteins were digested with Proteinase K, and immunoprecipitated DNA was eluted per manufacturer's instructions. The retrieved DNA was then subjected to amplification by quantitative real-time PCR using promoter-specific primers. Immunoprecipitated samples were normalized to respective input controls (10% of input sheared chromatin).

Statistical Analysis

Statistically significant differences between groups were assessed using the unpaired Student's T-test. Values for $p<0.05$ were considered significant. All experiments were performed a minimum of three times.

Results

Toxoplasma alone induces rapid and sustained phosphorylation and nuclear translocation of STAT1 in BMDC

In order for STAT1 to be active as a transcription factor, it must be phosphorylated on a key tyrosine residue (Tyr701) prior to translocation to the nucleus (51). To determine if parasites alone induce activation of STAT1, murine BMDC were infected with representative strains of the three primary clonal lineages of *Toxoplasma* (Type I – RH, Type II – PTG, Type III – M774.1), then fractionated into nuclear and post-nuclear extracts prior to immunoblot analysis with a phospho-STAT1-Tyr701 specific antibody (Figure 2.1A). Cells treated with IFNγ (100 ng/ml) served as a positive control. As expected, BMDC responded to IFNγ treatment with robust STAT1 phosphorylation and nuclear translocation, peaking at the early 30 minute time point. Unexpectedly, infection with *Toxoplasma* alone also induced phosphorylation and nuclear translocation of STAT1, independent of strain type. (Figure 2.1A). In contrast to IFNγ, *Toxoplasma*-mediated STAT1 phosphorylation was slower to develop, reaching peak levels at later timepoints. STAT1 phosphorylation declined over time with the type III strain M774.1 (Figure 2.1A), and this correlated with poor survival of that particular strain. Infection with another type III strain (CTG) resulted in sustained STAT1 activation (data not shown). Phosphorylation of a key serine residue (Ser727) is thought to be required for maximal transcriptional activity of STAT1 (25). Although not as strong as with IFNγ treatment, *Toxoplasma* infection was capable of inducing sustained STAT1 Ser727 phosphorylation (Figure 2.1A). Therefore, *Toxoplasma* infection alone induces activation of STAT1 and mediates its translocation to the nucleus.

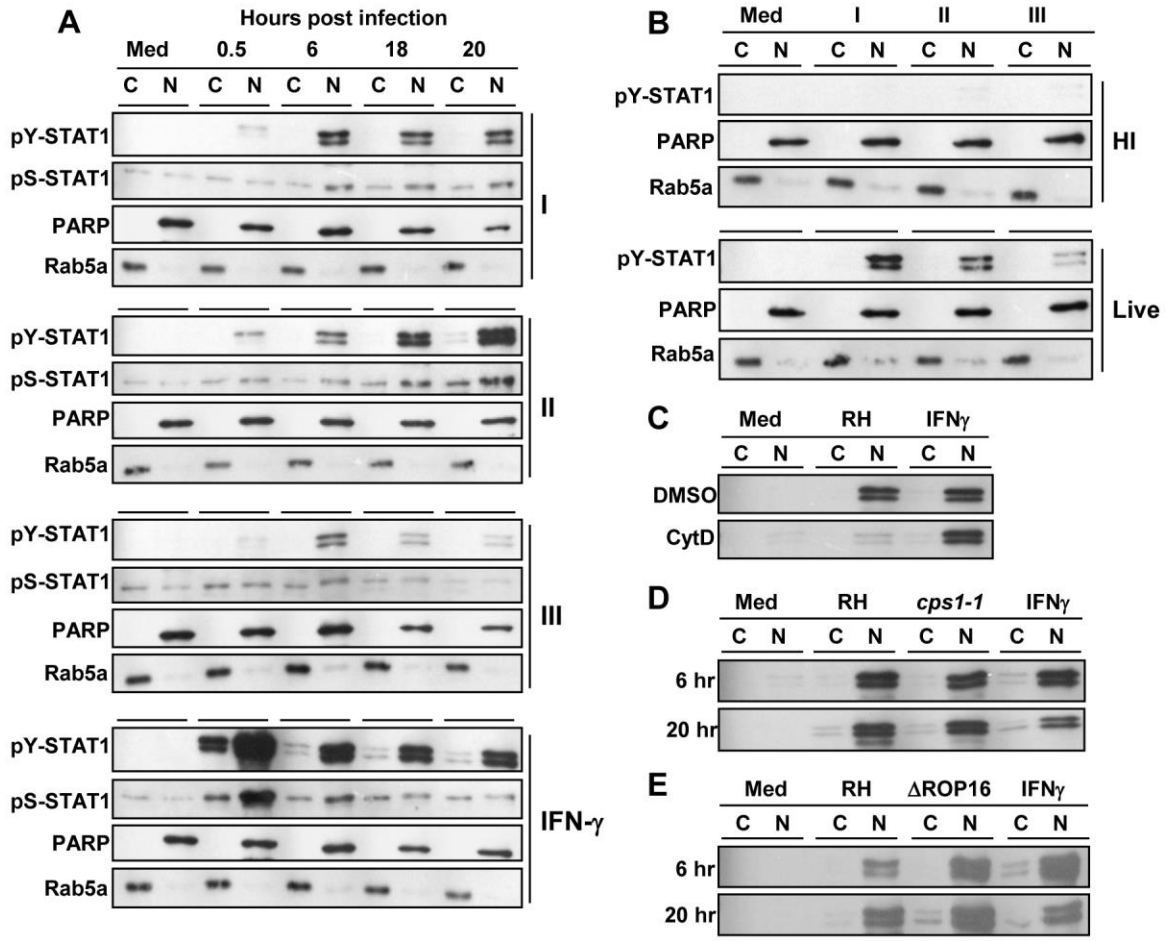

Figure 2.1. ***Toxoplasma*** **induces STAT1 phosphorylation and nuclear translocation in BMDC.** (A) BMDC were left in medium alone (Med), infected with type I (RH), II (PTG) or III (M774.1) strains of *Toxoplasma* (3:1 ratio of parasites to cells), or treated with murine IFNγ (100 ng/ml), prior to fractionation into cytoplasmic (C) and nuclear (N) extracts at the time points indicated. Samples were subjected to immunoblot analysis for phospho-Tyr701-STAT1 (pY-STAT1) and phospho-Ser-STAT1 (pS-STAT1). PARP and Rab5a served as loading controls for nuclear and cytoplasmic fractions, respectively. (B) Cells were infected with live parasites of the three strains as in (A) or exposed to heat-inactivated (HI) tachyzoites for six hours. Cytoplasmic and nuclear fractions were collected and immunoblot analyis was performed as in (A). (C) RH parasites were pre-treated for 10 min on ice with 1 μM cytochalasin D (CytD) prior to infection in the continued presence of the drug. Cells treated with the solvent DMSO alone served as controls. Cells were fractionated after six hours and subjected to immunoblot analysis for pY-STAT1. (D and E) BMDC were treated with IFNγ or infected with RH in comparison with either the *cps1-1* replication-deficient strain (D) or the ΔROP16 strain (E). Samples were fractionated after 6 and 20 hours and subjected to immunoblot analysis for pY-STAT1. All experiments were repeated at least three times with similar results.

STAT1 activation requires invasion by live parasites, and is independent of parasite replication and the rhoptry kinase ROP16

To further explore the mechanism by which parasites could trigger STAT1 phosphorylation, BMDC were infected with live parasites or treated with heat-inactivated tachyzoites prior to immunoblot analysis. As evident in Figure 2.1(B), heat-inactivated parasites were unable to initiate STAT1 phosphorylation. To confirm a requirement for active invasion, BMDC were infected with *Toxoplasma* in the presence of cytochalasin D, a drug that blocks actin polymerization and thereby also interferes with parasite gliding motility required for cell invasion (52). STAT1 phosphorylation and nuclear translocation were largely absent in the presence of the drug, whereas the response to IFNγ stimulation was unaffected (Figure 2.1C). Taken together, these results indicate a requirement for active invasion by live tachyzoites for parasite-mediated STAT1 activation.

Given the kinetics of parasite-induced STAT1 activation, with increasing levels of phosphorylated STAT1 detected at six hours and beyond, we next asked whether parasite replication could play a role. To address this question, a replication-deficient strain known as *cps1-1* was compared to the parental strain, RH, in terms of STAT1 activation. The *cps1-1* strain lacks an enzyme essential for uracil synthesis, and therefore parasite replication does not occur in uracil-free medium (50). BMDC were infected with either RH or *cps1-1* in the absence of uracil prior to immunoblot analysis. There was no detectable difference in STAT1 phosphorylation between the two strains (Figure 2.1D), indicating that replication of the parasite was not required. The requirement for invasion by live parasites for STAT1 phosphorylation implicated a parasite-derived secretory kinase. The rhoptry kinase ROP16 is known to directly phosphorylate STAT3

and STAT6 (53, 54) resulting in inhibition of proinflammatory cytokine production and promotion of arginase-1-dependent growth control (49, 55). We therefore asked whether STAT1 could be a collateral target of ROP16 by comparing BMDC infected with the parental RH strain with a ROP16-deleted strain (ΔROP16). As shown in Figure 2.1E, robust STAT1 phosphorylation was maintained even in the absence of ROP16.

STAT1 activation is confined to infected cells

We next asked whether the increasing amount of phosphorylated STAT1 over time occurred through secretion of a soluble DC factor in response to infection. To address this, supernatants from BMDC infected overnight were collected, centrifuged and filtered to remove parasites and cell debris, then transferred to uninfected BMDC. Cells treated with supernatants were compared with infected cells by immunoblot analysis of pY-STAT1. IFNγ treatment served as a positive control. Cells infected with each of the three parasite strains (I, II, III) or treated with IFNγ demonstrated the expected STAT1 phosphorylation response (Figure 2.2A). Transferred supernatant containing IFNγ was also capable of inducing STAT1 activation, but supernatant from infected cells was not sufficient, regardless of strain type (Figure 2.2B). From these results, we conclude that a soluble, secreted factor arising from infection is not involved in parasite-triggered STAT1 activation.

A closely related possibility was that parasite-initiated upregulation of a DC surface membrane-bound molecule might trigger STAT1 activation in bystander cells. To address this possibility, BMDC were infected with a low infection ratio of 0.5 parasites per cell overnight prior to flow cytometric analysis for pY-STAT1 expression (Figure 2.2C-E). The cell population was first

analyzed in terms of infection status (p30/SAG-1 positive, Figure 2.2C), then gated into separate uninfected (Figure 2.2D) and infected (Figure 2.2E) subpopulations to compare pY-STAT1 expression (blue line) versus the isotype control (red line). Unlike the uninfected group (Figure 2.2D), cells infected with RH showed a shift in pY-STAT1 expression relative to isotype control (Figure 2.2E), confirming that STAT1 phosphorylation is confined to infected cells.

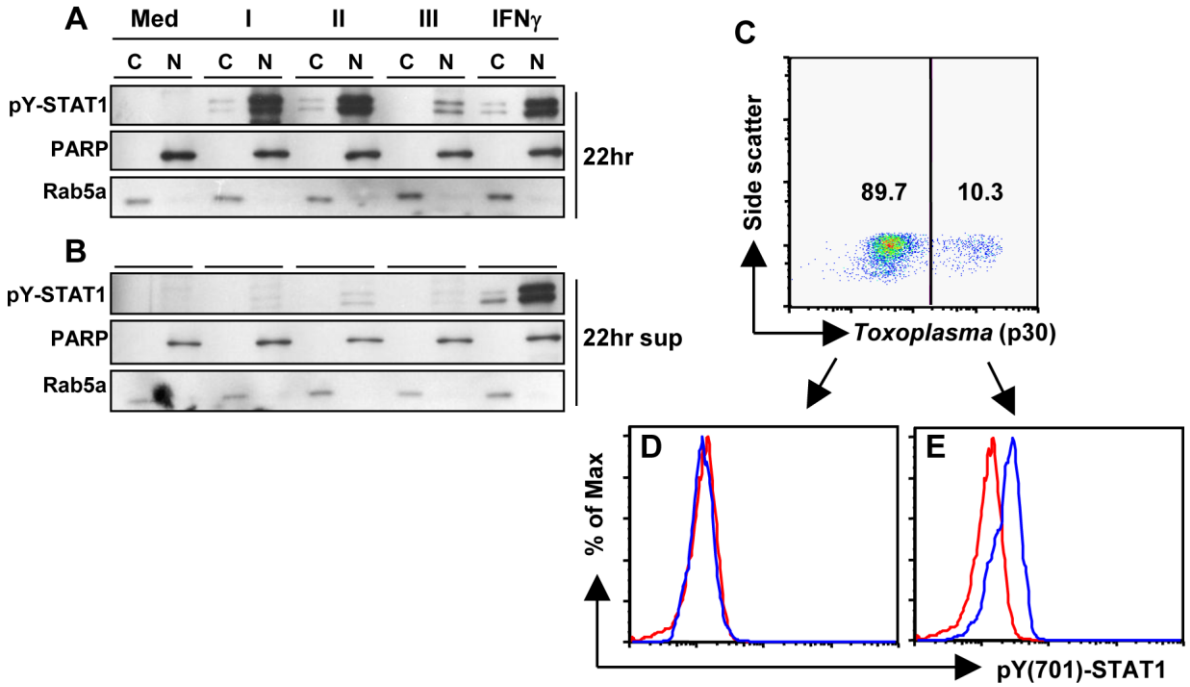

Figure 2.2. STAT1 phosphorylation is confined to infected cells. (A) BMDC were left in medium alone (Med), infected with type I (RH), type II (PTG) or type III (M774.1) strains of *Toxoplasma* (3:1 ratio of parasites to cells) or treated with murine IFNγ (100 ng/ml). After 22 hours supernatants were collected (22hr sup), centrifuged and filtered to remove debris and parasites, and subsequently transferred to additional untreated/uninfected BMDC for an additional 20 hours (B). For all samples (A and B), cytoplasmic (C) and nuclear (N) fractions were prepared and subjected to immunoblot analysis with phospho-Tyr701-STAT1 (pY-STAT1). PARP and Rab5a served as cytoplasmic and nuclear loading controls, respectively. (C - E) Cells were infected with the RH strain at a ratio of 0.5 parasites/cell for 20 hours, then subjected to intracellular staining for *Toxoplasma* (anti-p30/SAG-1) and pY-STAT1 prior to flow cytometric analysis. BMDC were gated on uninfected (D) and infected (E) populations to assess pY-STAT1 expression (blue lines) relative to staining with an isotype control antibody (red lines). All experiments were repeated at least twice with similar results.

Parasite-induced nuclear STAT1 binds IFN-γ-activated sequences (GAS) in vitro

Since *Toxoplasma* infection triggered STAT1 phosphorylation and nuclear translocation, we asked whether the activated STAT1 was indeed functional. When IFNγ-activated STAT1 homodimers translocate to the nucleus, they recognize and bind a palindromic IFNγ-activated consensus sequence (GAS) in the promoters of responsive genes in order to initiate transcription (56). To assess STAT1 binding, a transcription factor binding assay was performed whereby nuclear extracts from IFNγ-treated or parasite-infected BMDC were incubated with plate-immobilized STAT1 consensus sequences. Binding activity was subsequently detected using an anti-STAT1 antibody and quantified using an ELISA-based method. As shown in Figure 2.3, treatment with the positive control IFNγ or infection with the parasite strain RH (Tg) induced significant STAT1 binding activity. Addition of a competitive oligonucleotide (cOligo) reduced STAT1 binding to the medium control activity (set to 1), establishing binding specificity. Addition of a mutated oligo (mOligo) that could not compete for binding restored STAT1 binding activity to initial values in both cases. We conclude that *Toxoplasma*-triggered nuclear STAT1 is capable of recognizing and binding IFNγ-responsive consensus sequences.

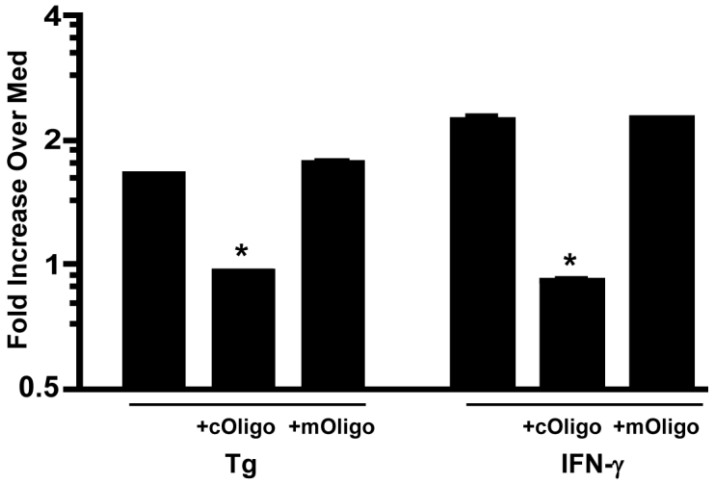

Figure 2.3. *Toxoplasma* induces *in vitro* STAT1 binding activity in BMDC. Nuclear extracts were prepared from cells infected with the RH parasite strain (Tg, *Toxoplasma gondii*; 3:1 parasites to cells) or treated with IFNγ (100 ng/ml) for 6 hours. The *in vitro* binding activity of nuclear STAT1 to solid phase IFNγ-activated sequence (GAS) oligonucleotides was assessed using an ELISA-based method. Binding activity is expressed as fold increase over cells cultured in medium alone (Med, value of 1). +cOligo, addition of soluble competitive oligonucleotides; +mOligo, addition of mutated non-competitive oligonucleotides. The experiment was repeated three times with similar results. *, $p < 0.05$ comparing nuclear extracts alone with nuclear extracts plus cOligo.

Toxoplasma and IFNγ together result in synergistic STAT1 phosphorylation and consensus sequence binding

The ability of *Toxoplasma* to induce STAT1 binding to consensus sequences was surprising, given that others have shown that the parasite blocks IFNγ-mediated STAT1-dependent activity (38, 39, 41, 42). To determine what impact the parasite may have on IFNγ-induced STAT1 phosphorylation, BMDC were pre-infected with *Toxoplasma* for two hours followed by subsequent IFNγ treatment. Cells were infected with *Toxoplasma* or treated with IFNγ alone for comparison, and analyzed by immunoblotting for pY-STAT1. *Toxoplasma* alone or IFNγ treatment alone triggered STAT1 phosphorylation and nuclear translocation as expected (Figure 2.4A). To our surprise, *Toxoplasma* and IFN-γ together provided a synergistic signal resulting in

greatly enhanced STAT1 phosphorylation. Synergistic STAT1 phosphorylation levels were maintained and even increased with IFNγ treatment over time (Figure 2.4A).

We next asked whether pre-infection could also enhance IFNγ-induced STAT1 binding to labeled gamma-activated sequence (GAS) oligonucleotides from the *Irf-1* promoter in an electrophoretic mobility shift assay (EMSA). Upon binding of its receptor, IFNγ triggers formation of a phosphorylated STAT1 homodimer known as gamma-activated factor (GAF) that is capable of binding to GAS elements in responsive promoters (51). As expected, 30 minutes of IFNγ stimulation resulted in strong binding to the labeled GAS oligonucleotides (Figure 2.4B). Addition of anti-STAT1 antibody but not normal rabbit IgG resulted in a supershift, indicating that the single complex contained STAT1, consistent with GAF (Figure 2.4C). When *Toxoplasma* alone was added to cells, no consistent shifts were detected (Figure 2.4B), although this is likely due to lack of assay sensitivity because oligonucleotide binding was observed in the transcription factor ELISA (Figure 2.3). Previous studies have indicated that *Toxoplasma* pre-infection reduces GAF formation in IFNγ-treated cells (32, 41). However, when BMDC were pre-infected for two hours and subsequently treated with IFNγ for 30 minutes, no reduction in GAF was observed (Figure 2.4B). Additionally, synergistic GAF formation was evident following IFNγ treatment for 2, 4 or 22 hours (Figure 2.4B). Interestingly, *Toxoplasma* in combination with IFNγ also induced an aberrant complex with lower electrophoretic mobility than GAF. We further explored this through a time course, noting that formation of this second complex increased over time, peaking at 22 hours (Figure 2.4B). Supershift experiments confirmed that the aberrant complex also contained STAT1 (Figure 2.4C). Taken together, these

data demonstrate that *Toxoplasma* pre-infection of BMDC leads to synergistic activation and binding of atypical STAT1 complexes to GAS sequences in response to IFNγ.

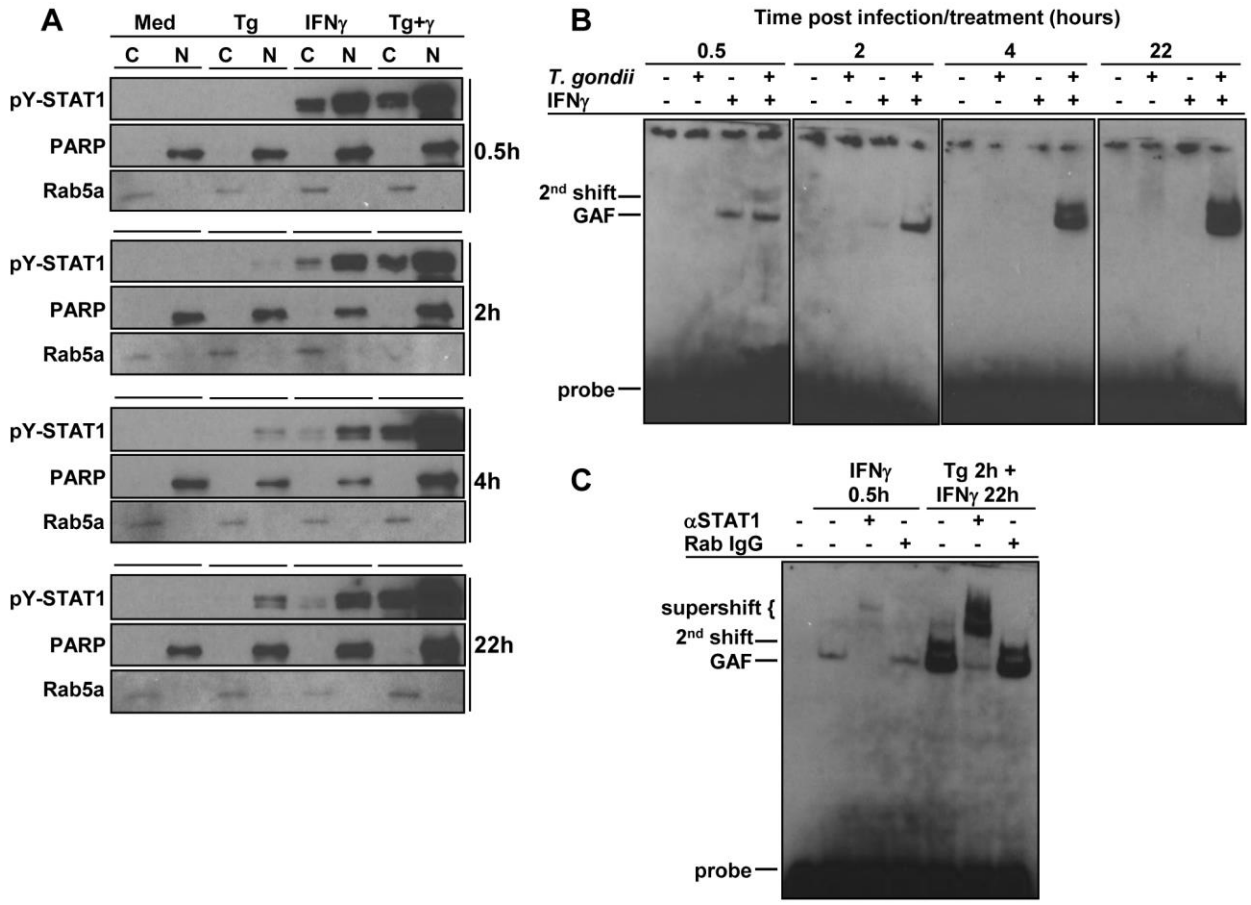

Figure 2.4. *Toxoplasma* synergizes with IFNγ in terms of phosphorylation and in vitro binding activity of STAT1. BMDC were left in medium alone (Med), infected with *Toxoplasma* (Tg, RH strain) at a ratio of 3 parasites per cell, treated with IFNγ (100 ng/ml), or infected with *Toxoplasma* followed 2 hours later by IFNγ treatment for the indicated times. In (A), cells were fractionated into cytosolic (C) and nuclear (N) extracts prior to immunoblot analysis for phospho-Tyr701-STAT1 (pY-STAT1). PARP and Rab5a served as loading controls for nuclear and cytoplasmic fractions, respectively. In (B) and (C), nuclear extracts were tested for binding to biotinylated probes containing the gamma-activated sequence (GAS) from the *Irf-1* promoter by EMSA. Supershift assays were also performed with samples in (C) using an antibody against STAT1α or normal rabbit IgG as a negative control. Experiments were repeated at least three times with similar results. GAF, gamma-activated factor (STAT1 homodimer).

***Toxoplasma* blocks IFNγ-induced, STAT1-dependent responses despite STAT1 activation**

Given the evidence of STAT1 activation and binding in *Toxoplasma*-infected BMDC, we hypothesized that in this cell type, tachyzoites may promote IFNγ/STAT1-dependent gene expression, even though in other cell types the parasite has the opposite effect. To address this, we utilized real-time quantitative PCR to examine the expression of known STAT1-dependent genes triggered by IFNγ stimulation, including the transcription factor interferon regulatory factor 1 (*Irf1)* (28, 29), and members of the p47 GTPase family known to be essential for survival during *in vivo* infection, *Lrg-47* and *Igtp* (17, 20, 57, 58). As expected, treatment with IFNγ resulted in induction of both *Irf1* (Figure 2.5A) and *Lrg-47* (Figure 2.5B), with peak fold induction over untreated samples at two hours post treatment. However, despite evidence of activated STAT1 in infected cells, *Toxoplasma* infection alone did not result in induction of the same genes (Figure 2.5A and B). Furthermore, when cells were first pre-infected for two hours followed by IFNγ stimulation, expression of the IFNγ-inducible target genes was blocked by presence of the parasite (Figure 2.5A and B). Expression of the p47 GTPase *Igtp* was also examined with similar results (data not shown).

To further explore the mechanism behind the parasite-mediated block in gene expression, we next asked whether activated STAT1 present in the nucleus could bind to the native promoter of an IFNγ responsive gene. *Toxoplasma* is known for the ability to interfere with permissive chromatin remodeling and thereby prevent binding of transcription factors to various promoters (41, 59, 60). To address this possibility in BMDC, we performed chromatin immunoprecipitation (ChIP) assays using an anti-STAT1 antibody. Precipitated DNA was subsequently amplified by quantitative PCR using primers specific for the *Irf1* promoter. As

anticipated, IFNγ induced strong STAT1 binding to the native *Irf1* promoter (Figure 2.5C). In contrast, pre-infection with *Toxoplasma* resulted in blockage of IFNγ-mediated binding while infection alone resulted in little to no binding (Figure 2.5C). Taken together with the gene expression results (Figure 2.5A and B), these data indicate that although activated STAT1 can bind target oligonucleotides (Figure 2.3, Figure 2.4B and C) and translocate into the nucleus of infected cells (Figure 2.1), transcriptional activity at STAT1-dependent, IFNγ-responsive genes remains blocked by the parasite. Therefore, our data reinforce the importance of an IFNγ-transcriptional blockade as an immune evasion mechanism in dendritic cells. We also demonstrate that the parasite itself triggers phosphorylation and nuclear translocation of STAT1. The function of STAT1 in the latter context remains to be determined.

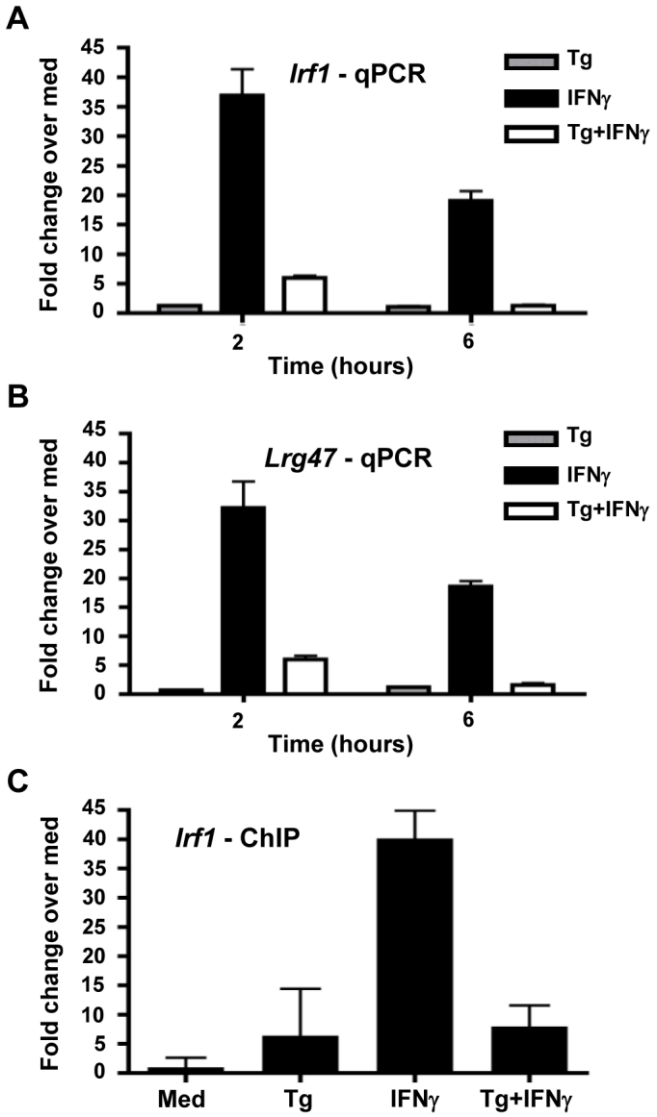

Figure 2.5. *Toxoplasma* blocks IFNγ-driven STAT1-dependent gene induction. BMDC were infected with the RH strain of *T. gondii* (Tg, ratio of 3 parasites/cell), treated with treated IFNγ (100 ng/ml), or pre-infected for 2 hours with RH prior to addition of IFNγ (Tg+IFNγ). At the indicated time points post cytokine treatment, total RNA was harvested and reverse-transcribed to cDNA prior to qPCR amplification of the IFNγ-responsive, STAT1-dependent genes *Irf-1* (A) and *Lrg-47* (B). Fold change in gene expression is expressed relative to BMDC in medium alone. Samples were normalized to the house-keeping gene GAPDH. To assess native chromatin binding, ChIP was performed using an anti-STAT1α antibody followed by qPCR amplification with primers specific for the *Irf-1* promoter (C). Experimental conditions are replicated as in (A and B), with a time point of 2 hours shown. In (C), fold change in promoter binding is expressed relative to untreated cells (Med). Samples were normalized to input chromatin. Experiments were repeated at least three times with similar results.

Discussion

Subversion of host immune responses, particularly those induced by the IFNγ/STAT1 signaling cascade, is now recognized as a key feature that contributes to the success of *Toxoplasma* as a parasitic organism. Although IFNγ is required for host defense, the response must also be partially counteracted to allow persistent life-long infection. Blockade of IFNγ-mediated transcription in infected cells as a means of immune evasion has been previously demonstrated in a range of cell types. In our study, we further highlight the prime importance of immune evasion in dendritic cells that are strategically situated at the front line of the host response to infection.

A key signaling intermediate downstream of IFNγ in anti-parasite immune responses is the transcription factor, STAT1. We initially anticipated that *Toxoplasma* would interfere with STAT1 activity in BMDC, either during the phosphorylation process as previously demonstrated in RAW264.7 cells (39) or by blocking downstream transcription as supported by others (36, 38, 40-42). Therefore, we were surprised to discover that *Toxoplasma* itself induces STAT1 phosphorylation and nuclear translocation. Concurrent with our work, another group recently published similar findings in human fibroblasts, confirming our results (42). We considered the possibility that STAT1 could be activated by the parasite rhoptry kinase ROP16, since that kinase is already known to phosphorylate STAT3 and STAT6 (53, 54). However, in contrast to the other group's findings (42), we found that parasite-induced STAT1 phosphorylation is not dependent on the rhoptry kinase ROP16 because we observed STAT1 activation in response to a Type II strain, which possesses an inactive ROP16 allele. Furthermore, a Type I strain in which ROP16 was genetically deleted retained STAT1 activation capability. The reason for this discrepancy is unclear, but could be due to the species and cell type differences between studies.

Nevertheless, we conclude that *Toxoplasma* induces ROP16-independent STAT1 phosphorylation and nuclear translocation in BMDC.

We do not yet know how STAT1 is activated by the parasite. However, we note that although ROP16 plays a role in maintaining STAT3 activation in infected cells, there is a substantial ROP16-independent STAT3 phosphorylation response during early infection (49). We considered whether this unknown ROP16-independent pathway could also be responsible for parasite-induced STAT1 phosphorylation. However, this scenario seems unlikely given that robust ROP16-independent STAT3 phosphorylation occurs very rapidly (within the first 15 minutes) whereas parasite-induced STAT1 phosphorylation increases gradually over time. In terms of STAT1 phosphorylation, active invasion by parasites is necessary. Yet, in the presence of cytochalasin D, a drug that prevents invasion while allowing discharge of rhoptries but not dense granules (61), STAT1 activation is prevented. This raises the possibility that a dense granule protein might activate STAT1, particularly insofar as dense granule protein GRA15 has recently been implicated in NFκB activation in the host cell (62). We note in that study activation of NFκB by GRA15 occurs with delayed kinetics, requiring ~4 hours after infection to achieve substantial p65 nuclear accumulation. The kinetics correlate with those we see during *Toxoplasma*-mediated STAT1 activation. We do not anticipate that GRA15 itself targets STAT1, given that only type II parasites express the active protein whereas we see strain-independent STAT1 phosphorylation, but nonetheless another dense granule protein could be responsible.

Interestingly, the combination of *T. gondii* and IFNγ synergized to stimulate potent STAT1 activation and nuclear translocation. It is not clear whether synergistic phosphorylation is

mediated via cross-talk of parasite and IFNγ-induced signaling pathways, or rather whether failure to dephosphorylate activated STAT1 could be the cause. It is possible that parasite infection down-regulates the IFNγ-induced expression of phosphatases such as suppressor of cytokine signaling 1 (SOCS1), known to negatively regulate STAT1 signaling. In this regard, IFNγ-mediated SOCS1 expression has been shown to be repressed by the parasite (40, 41). However, other data suggest that *Toxoplasma* induces SOCS1 expression in a ROP16-dependent manner (39, 55). Clearly, further work is required to determine the biologically relevant role, if any, of *Toxoplasma*-mediated interference of phosphatase activity.

In addition to synergistic STAT1 phosphorylation, we observed formation of an aberrant STAT1-containing complex, capable of binding to GAS oligonucleotides in vitro. A similar complex was noted by Lang et al in murine macrophages (41). In that study, *Toxoplasma* reduced IFNγ-mediated STAT1 homodimer formation by inducing formation of the aberrant complex. We did not observe a similar reduction and in fact both complexes increased over the 24 hour time course of infection. We also did not observe GAF formation in response to IFNγ at time points beyond 30 minutes by EMSA despite still being able to detect phosphorylated STAT1 in the nucleus by immunoblot analysis. We attribute this to a difference in assay sensitivity, given that IFNγ-mediated STAT1 phosphorylation is substantially reduced at later time points as part of a negative feedback response (Figures 2.1A and 2.4A). Assay sensitivity differences may also explain why we could detect an ~2-fold increase in oligonucleotide binding in response to IFNγ after 6 hours by the ELISA-based method (Figure 2.3) that was not seen later on with the EMSA method at a similar time point (Figure 2.4B). Regardless, an aberrant STAT1-containing complex did accumulate substantially over time in the parasite plus IFNγ-

treated group. Thus, it is possible that the additional parasite or host proteins involved may play a role in the mechanism of inhibition of IFNγ/STAT1-dependent gene transcription. Such a protein could allow binding of STAT1 to oligonucleotide sequences *in vitro*, but alter interactions with native chromatin and/or chromatin modifiers to prevent *in vivo* transcriptional activity of STAT1.

Our data corroborate previous studies with other cell types showing that *Toxoplasma* blocks the transcription of IFNγ-responsive, STAT1-dependent genes. This occurs despite the parasite's ability to activate STAT1. It is possible that *Toxoplasma*-activated STAT1 retains function in the regulation of a unique subset of genes, distinct from IFNγ-responsive, STAT1-dependent genes such as *Irf1* and the p47-GTPases. Indeed, a microarray study performed in bone marrow-derived macrophages identified a subset of IFNγ-responsive genes that were up-regulated by the presence of the parasite (41). That study was not performed in STAT1 null macrophages to determine whether STAT1 was required for the increase in transcription. It would be of interest in the future to perform microarray studies in infected BMDC in the presence and absence of STAT1 to determine what role, if any, *Toxoplasma*-activated STAT1 plays in the host response to infection.

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CHAPTER 3

Unraveling the Mechanisms of STAT1 Phosphorylation and Inhibition of IFNγ/STAT1-dependent Transcriptional Activity by *Toxoplasma gondii*[1]

[1] To be submitted for publication: Anne G. Schneider, Barbara A. Butcher, Barbara A. Fox, David J. Bzik and Eric Y. Denkers. Unraveling the mechanisms of STAT1 phosphorylation and inhibition of IFNγ/STAT1-dependent transcriptional activity by *Toxoplasma gondii*

Abstract

Evasion of the host immune response is critical to success for the protozoan parasite, *Toxoplasma gondii*. Critical to host defense is the antimicrobial cytokine, IFNγ. STAT1 is an important transcription factor responsible for many IFNγ-dependent antimicrobial effector functions. Although the parasite can block IFNγ-dependent STAT1 transcriptional responses, some STAT1 phosphorylation is induced by *Toxoplasma* alone, which is further enhanced by addition of IFNγ. How exactly the parasite inhibits IFNγ-dependent STAT1 activity, induces STAT1 phosphorylation, and causes synergistic STAT1 phosphorylation with IFNγ remains unknown. Here we show that the parasite inhibits STAT1 transcriptional activity independently of strain type in murine dendritic cells. The inhibition likely occurs independently of STAT3, despite evidence of STAT1/STAT3 association in infected cells. In investigating parasite-induced STAT1 phosphorylation further, we find the parasite rhoptry proteins ROP18 and 21 are not responsible. In addition, we exclude parasite-triggered G_iPCR, PI3K/AKT and MyD88-dependent TLR pathways in the mechanism of STAT1 phosphorylation and synergy with IFNγ. By contrast, we find that IFNγ-mediated induction of a negative regulator of IFNγ signaling, SOCS1, is repressed by the parasite, correlating with the observed synergistic STAT1 levels in the presence of IFNγ. These results serve to further enhance our knowledge about how *Toxoplasma* modulates STAT1 phosphorylation and transcription, pointing out new directions for future mechanistic studies.

Introduction

Approximately 30% of the world's human population is infected with the highly successful protozoan parasite, *Toxoplasma gondii* (1). For most healthy individuals, the infection is asymptomatic; however, consequences can be severe for the immuno-compromised (2). According to the Centers for Disease Control 2011 estimates for foodborne illness, this member of the phylum Apicomplexa ranks 2nd among pathogens causing foodborne-related deaths and 4th in terms of hospitalizations in the United States (3). In addition, this parasite causes severe abnormalities or death in neonates that acquired the infection *in utero* (4). Acute infections can be managed with a lengthy course of potentiated sulfa antibiotics, but potentially serious side effects can occur (5). In addition, the parasite can establish a life-long infection by the establishment of cysts within tissues, with potential to recrudesce later in life (6). Acquiring a better understanding of how this protozoan hijacks the host immune response is therefore of great interest in the design of future therapeutics and prophylactics.

Most *Toxoplasma* isolates found in people and domestic animals in North America can be assigned to three main clonal lineages, designated as type I, type II and type III (7). A fourth clonal type has also recently been described in North American wildlife (8, 9). The clonal lineage of the parasite is important as this can influence the ability of the *Toxoplasma* to modulate the host's immune response and thereby impact the clinical outcome of infection. In mice, type I strains are universally lethal during acute infection, whereas type II and III strains are much less virulent and are capable of establishing chronic infection (10). Although type II strains are most commonly isolated from people in North America, type I strains have been implicated more frequently in cases of ocular disease (11). Despite high sequence similarity, the

three strains differ in some key proteins that are secreted into the host cell upon invasion. ROP16 is an example of key rhoptry protein known to regulate the host immune response. ROP16 from type I and III strains phosphorylates STATs 3 and 6 (12, 13), leading to inhibition of pro-inflammatory cytokine signaling and promotion of arginase-1-dependent growth control (14, 15). Additional strain-dependent parasite molecules shown to modulate host immune responses include ROP18 (reviewed in (16)), ROP5 (17-21), and the dense granule protein, GRA-15 (15, 22, 23). These mechanisms and others for inhibition of host responses become vital as the parasite is known to trigger host signaling pathways including several toll-like receptors as well as G_iPCRs, PI3K/AKT, and MAPKs (reviewed in (24)). The balance between pathogen detection and immune evasion helps determine survival of the pathogen versus the host.

Among the most important immune system components targeted by *Toxoplasma* is the interferon gamma (IFNγ) response to the parasite. This cytokine, produced largely by T cells and NK cells, is critical to controlling parasite replication through various antimicrobial mechanisms in both mice and humans (25-30). IFNγ exerts its effects through activation of a Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway. Briefly, binding of IFNγ to its receptor stimulates phosphorylation of key JAK molecules (JAKs 1 & 2), resulting in JAK-mediated phosphorylation of the receptor. STAT1 is then recruited to the receptor and is in turn phosphorylated on a key tyrosine residue (Y^{701}). STAT1 homodimers form and translocate to nucleus where they initiate gene transcription (31-33). One of the genes induced in a STAT1-dependent manner is interferon regulatory factor-1 (*Irf1*), itself a transcription factor that induces transcription of additional genes (34, 35). STAT1 has been shown to be critical to survival of

acute *Toxoplasma* infection (36, 37), while mice deficient in IRF1 also demonstrate increased susceptibility (38). Thus, the IFNγ/STAT1 pathway is a prime target for immune-evasion by the parasite.

Toxoplasma has previously been shown to block IFNγ-mediated gene expression at the level of key individual genes, such as *Irf1*, as well as globally as determined by microarray analysis (23, 39-50). This inhibitory capacity has been identified in a variety of cell types including bone marrow-derived macrophages (BMDM), dendritic cells (BMDC), human fibroblasts, the RAW264.7 monocyte/macrophage cell line, astrocytes, and microglia. Recent studies implicate a mechanism involving impairment of transcription factor binding within the nucleus, likely involving regulators of chromatin remodeling (23, 46, 49, 50). In those studies, IFNγ-induced STAT1 phosphorylation remained intact in the presence of parasite. However, one study in RAW264.7 cells indicated that parasite-mediated expression of suppressor of cytokine signaling-1 (SOCS1), a key regulator of IFNγ/JAK/STAT1 signaling, could block IFNγ-induced STAT1 phosphorylation (47). In contrast, others show *Toxoplasma* can block IFNγ-mediated upregulation of *Socs1* expression (48, 49). In addition, we and others have recently shown that the parasite infection alone can trigger STAT1 phosphorylation (23, 50), which is enhanced by the subsequent addition of IFNγ (50). Despite this, transcriptional responses to IFNγ remain blocked by the parasite. The parasite molecules involved in STAT1 phosphorylation and inhibition of IFNγ-dependent transcription remain unknown.

In this study, we sought to further unravel the mechanisms behind parasite-mediated inhibition of IFNγ-induced STAT1 transcriptional activity, parasite-induced STAT1 phosphorylation, and the

reason for synergistic STAT1 phosphorylation when combined with IFNγ. As in our previous study, we utilized bone marrow-derived dendritic cells. DCs are a key cell type in the early immune response to *Toxoplasma* infection, participating in immune activation as well as dissemination of the parasite throughout the host (51-54). This importance is further highlighted by studies where mice depleted of DCs succumb to acute infection (55, 56). Previously, we confirmed that parasite-mediated inhibition of STAT1 transcriptional activity also occurs in DCs (50). Here we show that this inhibition occurs independently of parasite strain type and likely without STAT3 involvement, despite evidence of STAT1/STAT3 interaction in infected cells. We also previously demonstrated that the STAT3/6-directed parasite kinase ROP16 did not mediate STAT1 activation (50). Here we show that the rhoptry kinases ROP18 and the presumptive kinase ROP21, are also not required for STAT1 phosphorylation. Further, we found no evidence for involvement of parasite-triggered MyD88-, G_iPCR- or PI3K/AKT-dependent pathways for either parasite-induced STAT1 phosphorylation or synergy with IFNγ. Interestingly, infection alone induced modest *Socs1* induction that was dependent upon ROP16. However, IFN-γ-mediated *Socs1* induction was blocked by *Toxoplasma* regardless of the presence or absence of functional ROP16. Impaired expression of this negative regulator of IFNγ signaling by the parasite correlated with the synergistic phospho-STAT1 levels observed. These findings provide additional information that will provide new directions in defining the precise mechanisms at play.

Materials & Methods

Ethics Statement

All experiments were performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The Institutional Animal Care and Use Committee (IACUC) at Cornell University (permit number 1995-0057) approved all protocols prior to use. Animal suffering was minimized to the greatest extent possible.

Mice and Parasites

Female C57BL/6 mice of 6-8 weeks of age were purchased from either Taconic Farms or the Jackson Laboratory. Female myeloid differentiation primary response gene 88 (MyD88) knock-out mice (on a partially backcrossed background – 129/Ola x C57BL/6), originally generated by Akira et al (57), were kindly provided by Ling Qi (Cornell University). All mice were subsequently housed at the Transgenic Core Mouse Facility (TMCF) under specific pathogen-free conditions at Cornell University's College of Veterinary Medicine, which is accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC). The following *Toxoplasma gondii* strain tachyzoites were maintained by bi-weekly passage in human foreskin fibroblast (HFF; the American Type Tissue Collection) monolayers in DMEM (Life Technologies) supplemented with 1% bovine growth serum (Hyclone), 100 U/ml penicillin (Life Technologies) and 0.1 mg/ml streptomycin (Life Technologies): RH (type I), PTG (type II), CTG (type III), a ROP18-deleted strain ($\Delta ROP18_I$), a ROP21-deleted strain ($\Delta ROP21_I$) and a ROP16-deleted strain on an RH background ($\Delta ROP16_I$). The rhoptry deleted strains were constructed as previously described (14, 58). Parasite cultures were routinely tested every 6-8

weeks using a commercial PCR/ELISA-based kit (Roche diagnostics) for *Mycoplasma* contamination.

Bone Marrow-Derived Dendritic Cell (BMDC) Culture

A single cell suspension was prepared from marrow that was flushed from a femur and tibia of a C57BL/6 mouse and reconstituted in BMDC "low" medium consisting of RPMI 1640 (Fisher Scientific) supplemented with 100 U/ml penicillin (Life Technologies), 0.1 mg/ml streptomycin (Life Technologies), 10% fetal calf serum (Hyclone), 50 μM of 2-mercaptoethanol (Sigma) and 20 ng/ml of granulocyte/monocyte-colony stimulating factor (GM-CSF, Peprotech). Cells were plated on 100 x 15 mm sterile polystyrene Petri dishes (Fisher Scientific) and incubated at 37°C at 5% CO_2 for a total period of 9 days. On day 3, BMDC were supplemented with fresh BMDC "low" medium. On day 6, cells were supplemented with BMDC "high" medium, which contained 50 mM of 2-mercaptoethanol (Sigma). On day 8, GM-CSF alone was added at 200 ng per plate. The non-adherent cells (BMDC) were harvested on day 9, counted, and plated at 3 x 10^6 for in vitro infections or cytokine stimulations unless otherwise specified. Cells were plated in complete medium (cDMEM) consisting of DMEM (Life Technologies) supplemented with 10% bovine growth serum (Hyclone), 50 μM 2-mercaptoethanol (Sigma) and the following reagents from Life Technologies: 0.1 mg/ml streptomycin, 100 U/ml penicillin, 3% HEPES, 0.1 mM non-essential amino acids, and 1mM sodium pyruvate.

***In vitro* Infections, Stimuli, and Inhibitor Treatments**

Unless otherwise noted, *in vitro* infections of BMDC with *Toxoplasma* were performed by the addition of 3 parasites per cell. Synchronization of parasite contact with cells was achieved by

brief centrifugation (200 x g, 3 min). For some samples, cells were treated with 100 ng/ml of recombinant interferon-gamma (IFNγ, Peprotech). In some cases, BMDC were first pre-infected with *Toxoplasma* for 2 hours, followed by IFNγ stimulation for varying time points. For the inhibitor experiments, BMDC were first pre-treated with 50 ng/ml of pertussis toxin (Sigma) or 50 ng/ml of Wortmannin (Sigma) prior to infection or treatment with IFNγ.

Immunoblot Analysis

Cells (3 x 10^6 per sample) were fractionated into cytoplasmic and nuclear fractions using a nuclear extract kit (Active Motif) as indicated. Samples were diluted with 2x SDS sample reducing buffer and boiled for 5 minutes. Proteins were separated by 10% SDS-PAGE, followed by electro-transfer to nitrocellulose membranes (Whatman). Membranes were blocked for 1 hour at room temperature in Tris-buffered saline containing 0.1% Tween-20 at pH 7.6 (TBST) supplemented with 5% nonfat dry milk. Washes with TBST were performed prior to incubation of membranes overnight at 4°C in primary antibody diluted in TBST with 5% bovine serum albumin (Calbiochem). Primary antibodies used included the following (all purchased from Cell Signaling): anti-phospho-STAT1-Tyr-701 (catalog no. 9167), anti-phospho-STAT3-Tyr-705 (catalog no. 9131), anti-PARP (catalog no. 9542) and anti-Rab5a (catalog no. 2143). Membranes were washed again prior to incubation with horseradish peroxidase-conjugated anti-rabbit IgG (Jackson ImmunoResearch, catalog no. 111-035-144) in blocking buffer. After additional wash steps, protein bands were observed using a chemiluminescent detection system (Thermo Scientific).

Quantitative Reverse Transcriptase PCR (qPCR)

BMDC were infected with parasite or treated with cytokine for varying time points prior to harvesting samples. Total RNA was isolated using the E.Z.N.A Total RNA Miniprep kit (Omega Bio-Tek), which included an on-column DNase digestion step (Agilent Technologies) to remove any residual DNA. cDNA was synthesized from total RNA using qScript cDNA SuperMix (Quanta Biosciences). Quantitative PCR was performed using cDNA as the template using SYBR Green chemistry (Quanta Biosciences) and ABI Prism 7500 sequence detection system (Applied Biosystems). The relative expression of target genes in the experimental samples were compared to medium alone control samples using the $\Delta\Delta C_T$ method, after normalization of the target genes to the house-keeping gene GAPDH. The primer sequences used were *Irf1* forward: 5'-TTGGCATCATGGTGGCTGT-3'; *Irf1* reverse: 5'-AAGGAGGATGGTCCCCTGTTT-3'; *Socs1* forward: 5'- TGTAGCAGCTTGTGTCTGG-3'; *Socs1* reverse: 5'- CCTGGTTTGTGCAAAGATACTG-3'; *Gapdh* forward: 5'-AATGGTGAAGGTCGGTGTG-3', *Gapdh* reverse: 5'-GTGGAGTCATACTGGAACATGTA-3' (all primers were purchased from IDT Technologies).

Co-Immunoprecipitation Assay

BMDC were infected with parasites or stimulated with cytokine for the desired time periods. Cells were then harvested, wash once in cold PBS and resuspended in 1x lysis buffer (Cell Signaling) supplemented with 1 mM fresh PMSF. Samples were incubated for 10 minutes on ice prior to brief sonication for 3 pulses of 5 seconds each. Cell lysates were then microcentrifuged for 10 minutes at 14,000 x g to remove cellular debris and then incubated with primary antibody overnight (1:100 dilution). Primary antibodies were the same as used for immunoblotting.

Protein A/G agarose beads (Thermo Scientific) were added to each sample and incubated for 2 hours at 4°C. The beads were washed five times with 1x cell lysis buffer, then resuspended in 2x SDS reducing sample buffer and boiled to release the antibody complexes. Beads were pelleted and the supernatants were subjected to immunoblot analysis.

Electrophoretic Mobility Shift Assay (EMSA)

To confirm STAT1 binding to appropriate oligonucleotide probes in nuclear extracts, a chemiluminescent EMSA assay was performed using a kit (LightShift kit; Thermo Scientific) as indicated. Briefly, nuclear extracts were incubated for 20 minutes with 200 fmol of biotinylated probe designed to include the gamma-activated sequence (GAS) from the *Irf1* promoter. The probe consisted of complementary oligonucleotides (5'-CATTTCGGGGAAATCGATC-3' and 5'-GATCGATTTCCCCGAAATG-3'; IDT Technologies) labeled at the 5' ends with biotin that were subsequently annealed in equimolar quantities. Other binding components in the reaction included binding buffer (Thermo Scientific), 1 μg poly(I)-poly(C), 1 mM DTT, 1 mM EDTA, 2.5% glycerol and 50 mM KCl. In the case of supershift experiments, 2 μg of the appropriate antibody was added to the reaction mixture for 20 minutes on ice prior to the addition of the nuclear extract for an additional 20 minutes at room temperature. Supershift-grade antibodies were purchased from Santa Cruz Biotechnology and included rabbit anti-STAT1α p91 (catalog no. sc-591x) and rabbit anti-STAT3 (clone k-15, catalog no. sc-483), while normal rabbit IgG served as a negative control (catalog no. sc-2027). Resolution of protein-DNA complexes was performed via native gel electrophoresis using 5% polyacrylamide gels (Bio-Rad) in 0.5x TBE buffer. The complexes were electrotransferred to a positively-charged nylon membrane followed by cross-linking of the DNA to the membrane using a commercial UV light cross-linking

instrument (UV Stratalinker 2400, Stratagene) at 120 mJ/cm^2 using the auto cross-link function. Gel shifts were detected using streptavidin-horseradish peroxidase conjugate and chemiluminescent substrate (Thermo Scientific).

Statistical Analysis

Statistically significant differences between experimental groups were determined using the unpaired Student's t-test. A difference was considered statistically significant if $p < 0.05$. Experiments were performed at least three times.

Results

Inhibition of IFNγ-driven STAT1 transcriptional activity occurs independently of *Toxoplasma* strain type

We and others have previously documented the ability of the parasite to inhibit IFNγ-driven gene expression (23, 46, 48-50). Although inhibition is thought to involve modulation of chromatin remodeling, further details of the mechanism remain unclear. To address this further, we infected BMDC with representatives of the three clonal lineages of *Toxoplasma* to determine whether parasite strain type was an important key to inhibition. Previously, inhibition was found to occur independently of strain type in a HEK293 cell line transduced with a stable GAS reporter and in RAW264.7 cells by microarray analysis (23). We assessed inhibition of IFNγ-induced expression of interferon regulatory factor 1 (*Irf1*) by qPCR after pre-infection with the different parasite strains. None of the parasite strains induced significant *Irf1* expression on their own, whereas IFNγ triggered ~20-22 fold increase (Figure 3.1A). When cells were pre-infected prior to IFNγ addition, both type I and type II strains significantly reduced *Irf1* expression with no significant difference noted between strains (Figure 3.1A). Further evidence of a strain-independent response was apparent when comparing the type I strain with the ROP16-deleted strain. As seen in Figure 3.1B, inhibition of *Irf1* expression did not require the presence of ROP16. A type III strain (CTG) was also examined; although this strain could block *Irf1* transcription in some experiments, the inhibition was not always consistent (data not shown). We conclude that inhibition of IFNγ-driven STAT1-dependent transcriptional activity does not rely on strain-type differences.

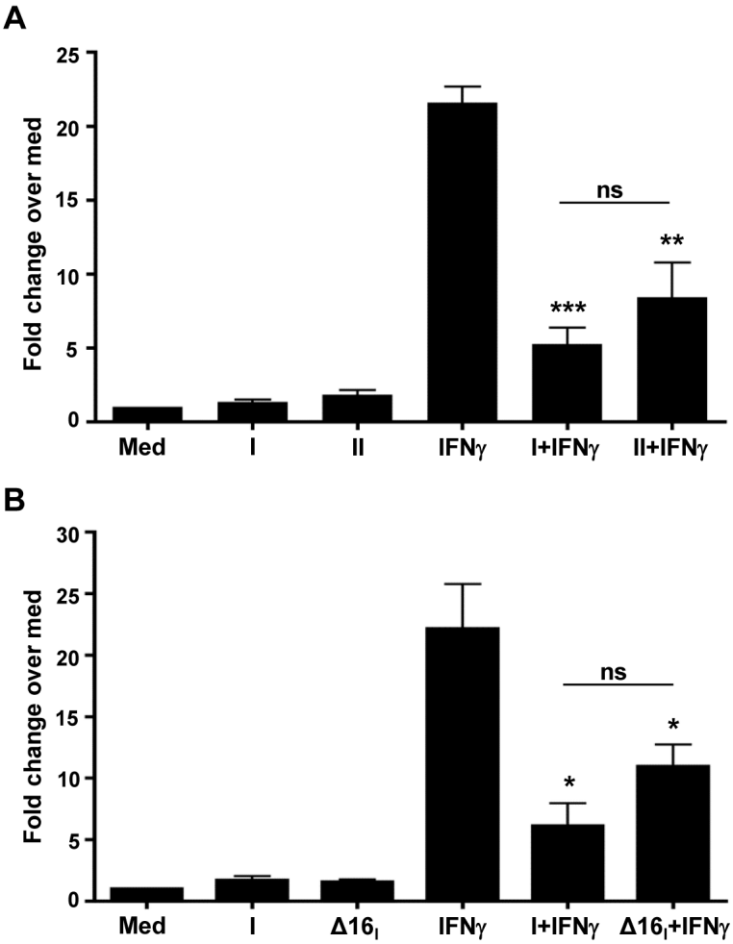
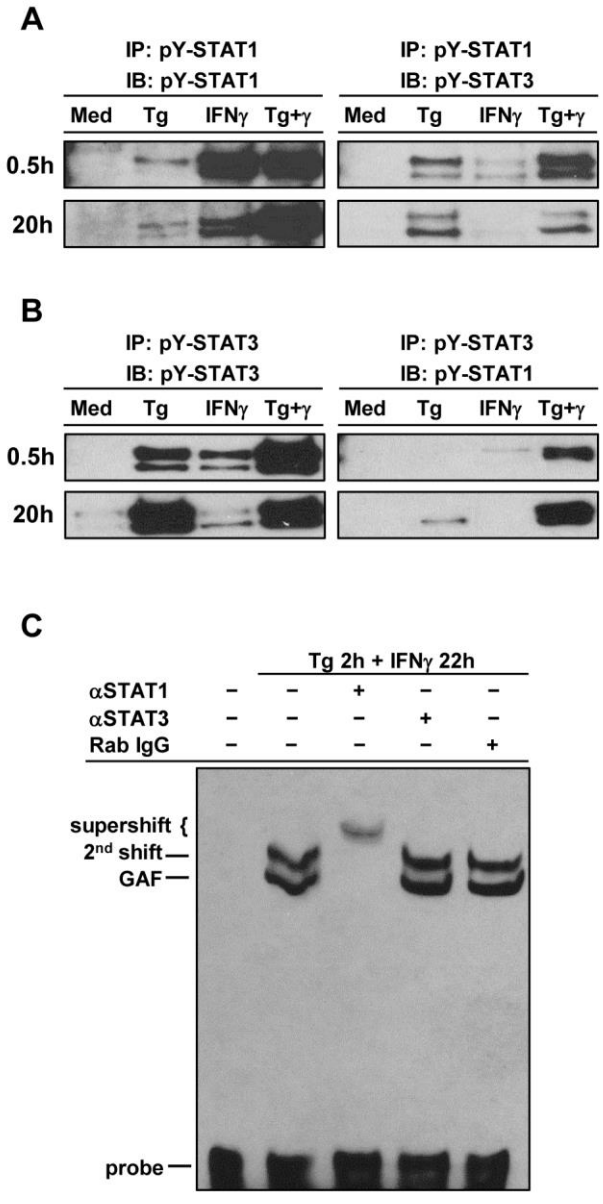


Figure 3.1. *Toxoplasma*-mediated inhibition of IFNγ-induced *Irf1* expression occurs in a parasite strain-independent manner. (A) Comparison of type I versus type II strains in the ability to inhibit *Irf1* expression. BMDC were treated with medium alone (Med), IFNγ (100 ng/ml), or infected with a type I (RH) or type II (PTG) strain of *Toxoplasma* at a ratio of 3 parasites per cell for 2 hours. Some samples were first pre-infected with parasite for 2 hours, followed by IFNγ treatment for an additional two hours (I+IFNγ, II+IFNγ). Total RNA was prepared and reverse-transcribed to cDNA prior to qPCR analysis for the *Irf1* gene. Fold change in gene expression is plotted relative to medium alone samples (set to 1). Samples were normalized to the house-keeping gene GAPDH. (B) Comparison of a ΔROP16 strain with the parental type I (RH) strain. Experiment was performed as in (A) with the exception of the parasite strain types used (type I – RH versus $\Delta16_I$ – ΔROP16 on an RH background). Experiments were performed three times with similar results. * = $p < 0.05$; ** = $p < 0.01$, *** = $p < 0.001$ when compared to IFNγ treatment alone. Ns, not significant.

Despite evidence of STAT1/STAT3 association, STAT3 does not contribute to aberrant complex formation

In thinking further about an inhibition mechanism, we considered that the parasite is able to induce phosphorylation of both STAT1 and STAT3. Although partially dependent on ROP16, there is also some ROP16/strain-independent STAT3 phosphorylation that occurs in infected cells that could potentially play a role (14). In addition, there was some evidence in the literature that STAT3 can associate with STAT1 and lead to impairment of some STAT1-pro-inflammatory responses (59-61). To investigate this further in dendritic cells, we performed co-immunoprecipitation assays to assess STAT1/STAT3 interactions in infected cells. When cell lysates were immunoprecipitated and then immunoblotted for pY-STAT1, we could detect pY-STAT1 as expected in parasite alone, IFNγ-treated, and parasite plus IFNγ treated groups for both time points (Figure 3.2A). When the same samples were immunoblotted for pY-STAT3, we successfully pulled down STAT3 in both *Toxoplasma* alone and parasite plus IFNγ groups. A small amount of STAT3 could be detected with IFNγ alone at 30 minutes that subsequently disappeared by 20 hours (Figure 3.2A). This is not completely unexpected as some STAT3 phosphorylation in response to IFNγ has been previously reported by others (62). The reverse co-immunoprecipitation experiment was performed to confirm the STAT1/STAT3 interaction. As shown in Figure 3.2B, after immunoprecipitation for pY-STAT3, we were able to pull down pY-STAT1 in response to parasite alone at 20 hours, as well as in the parasite plus IFNγ group for both time points. Thus, we were able to show an association between STAT1 and STAT3 in infected cells, particularly with additional IFNγ treatment, but whether that association was functionally relevant required further investigation.

Figure 3.2. STAT3 associates with STAT1 in infected cells, yet does not contribute to parasite-induced aberrant STAT1 complex formation. (A) BMDC were treated with medium alone (Med), IFNγ, or infected with *Toxoplasma* (Tg, RH strain) at ratio of 3 parasites per cell for the time points indicated. For some samples, cells were first pre-infected with parasite for 2 hours followed by treatment with IFNγ for the specified time periods (Tg+γ). Cells were lysed and subjected to immunoprecipitation (IP) using an antibody against phospho-STAT1-Tyr701 (pY-STAT1). pY-STAT1 immunoprecipitated samples were then analyzed by immunoblot analysis (IB) for pY-STAT1 as a control, and phospho-STAT3-Tyr705 in a co-immunoprecipitation assay. (B) Experiment was performed as in (A), except that samples were initially immunoprecipitated with an anti-pY-STAT3 antibody, followed by immunoblot analysis for both pY-STAT3 and pY-STAT1. (C) BMDC were treated with medium alone or first pre-infected with *Toxoplasma* for 2 hours prior to IFNγ addition for 22 hours (Tg 2h + IFNγ 22h). At that time, nuclear extracts were prepared and subjected to analysis by a chemiluminescent electrophoretic mobility shift assay (EMSA). A biotinylated probe containing a gamma-activated sequence (GAS) from the *Irf1* promoter was utilized. Supershift assays were also performed using antibodies directed against STAT1 (αSTAT1), STAT3 (αSTAT3), or normal rabbit IgG (rab IgG) as a negative control. GAF, gamma-activated factor (STAT1 homodimer). Experiments were performed three times with similar results.


A
IP: pY-STAT1
IB: pY-STAT1
IP: pY-STAT1
IB: pY-STAT3
Med
Tg
IFNγ
Tg+γ
0.5h
20h
B
IP: pY-STAT3
IB: pY-STAT3
IP: pY-STAT3
IB: pY-STAT1
C
Tg 2h + IFNγ 22h
αSTAT1
αSTAT3
Rab IgG
supershift {
2nd shift
GAF
probe

We and others had previously shown that *Toxoplasma* can induce the formation of a gamma-activated site (GAS)-binding aberrant STAT1-containing complex by EMSA (49, 50). We speculated that whatever was binding to STAT1 in the abnormal complex could play a role in inhibition of IFNγ-driven transcriptional responses. Given the evidence in Figures 3.2A-B that STAT3 could associate with STAT1 in infected cells, we hypothesized that STAT3 could be contributing to the aberrant complex formation. To address this, we infected cells for 2 hours with *Toxoplasma* followed by IFNγ stimulation for 22 hours, as these conditions induced the most robust aberrant complex formation in the previous study. An EMSA assay was performed, with assessment of *Irf-1* GAS sequence binding indicated by the presence of gel shifts. In the absence of any additional antibody, two distinct gel shifts were observed as expected – one consistent with gamma-activated factor (GAF, STAT1 homodimer) and a second aberrant complex of lesser mobility (Figure 3.2C). Addition of an αSTAT1 antibody supershifted both bands, as expected, whereas the rabbit IgG control antibody had no effect (Figure 3.2C). However, when an αSTAT3 antibody was added instead, a supershift was not detected (Figure 3.2C). Therefore, although STAT1 can associate with STAT3 in infected cells, this association does not explain the aberrant complex formation potentially involved in inhibition of IFNγ-mediated transcription. Attempts were also made to identify unique protein bands for further analysis in infected cells following STAT1 immunoprecipitation (data not shown). However, such attempts were unsuccessful, leaving the identity of the mysterious binding partner yet to be discovered.

STAT1 phosphorylation induced by *Toxoplasma* does not require the parasite rhoptry kinases ROP18 and ROP21

Previously we had shown that the parasite rhoptry kinase ROP16, although already known to induce STAT3 phosphorylation, was not required for parasite-mediated STAT1 phosphorylation in BMDC (50). This did not, however, rule out involvement of other rhoptry kinases. Given our access to the deleted rhoptry kinase strains ΔROP18 and ΔROP21, we investigated whether these parasite proteins could contribute to the mechanism of parasite-mediated STAT1 phosphorylation. ROP18 has been identified in forward genetic screens as a key virulence determinant known to phosphorylate certain immunity-related GTPases (IRGs), thereby reducing their accumulation at the parasitophorous vacuole membrane (in coordination with another key virulence determinant, ROP5) and consequently preventing parasite destruction (reviewed in (16)). In addition, this parasite kinase has been shown to mediate degradation of the host endoplasmic reticulum-resident transcription factor activating transcription factor 6 beta (ATF6β), leading to impairment of initiation of CD8 T cell adaptive immune responses (63, 64). ROP21 is of unknown function, although its nucleotide acid sequence predicts it to be an active kinase. While ROP21 has not been identified as a parasite virulence determinant thus far, it has been shown to localize to the parasitophorous vacuole membrane, and later during in infection, the host cytosol (65). To determine whether these rhoptry kinases could be involved in STAT1 phosphorylation, we performed *in vitro* infections in BMDC. Nuclear and cytoplasmic extracts were assessed for STAT1 tyrosine phosphorylation in cells infected with the wild-type RH strain versus the ROP18- and ROP21-deleted strains. RH parasites and the positive control IFNγ induced STAT1 tyrosine phosphorylation as expected in both experiments, at both the 6 hour and 22 hour time points (Figure 3.3A, B). However, there were no discernible differences in STAT1

phosphorylation when comparing the RH strain to either the ΔROP18 (Figure 3.3A) or ΔROP21 (Figure 3.3B) strains at either time point examined. Therefore, we conclude that in addition to ROP16, neither ROP18 nor ROP21 are required for *Toxoplasma*-induced STAT1 tyrosine phosphorylation.

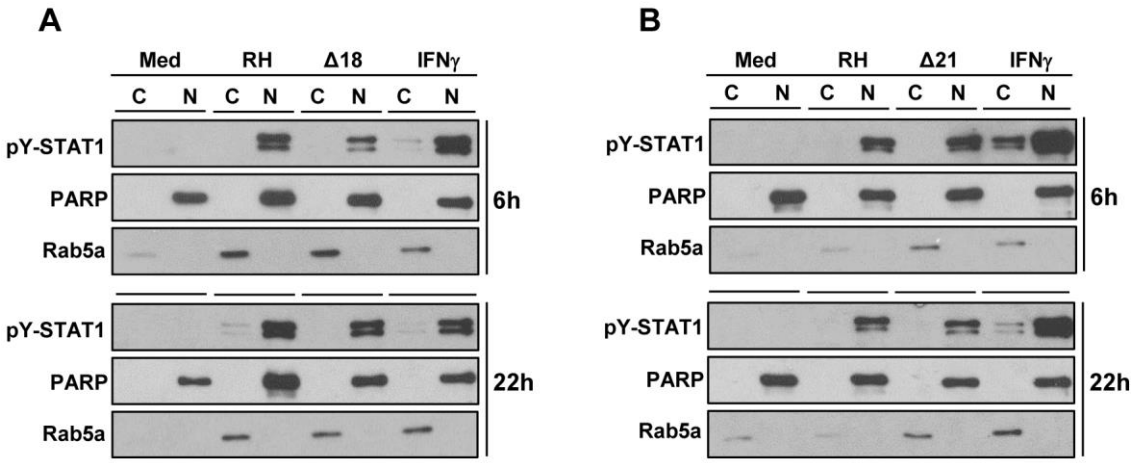


Figure 3.3. ROP18 and ROP21 are not required for *Toxoplasma*-induced STAT1 phosphorylation. (A) BMDC were left in medium alone (Med), treated with IFNγ (100 ng/ml), or infected with RH (type 1 parasite) or the ROP18-deleted strain (Δ18) at a 3:1 ratio of parasites to cells. After 6 or 22 hours, cytoplasmic and nuclear extracts were prepared and subjected to immunoblot analysis for phospho-STAT1-Tyr701 (pY-STAT1). PARP and Rab5a served as nuclear and cytoplasmic loading controls, respectively. (B) BMDC were treated as in (A), with the exception that infection with RH was compared to that of a ROP21-deleted strain (Δ21). Experiments were performed twice with similar results.

Parasite-mediated STAT1 phosphorylation and synergy with IFNγ do not require MyD88-, G_iPCR- or PI3K-dependent pathways

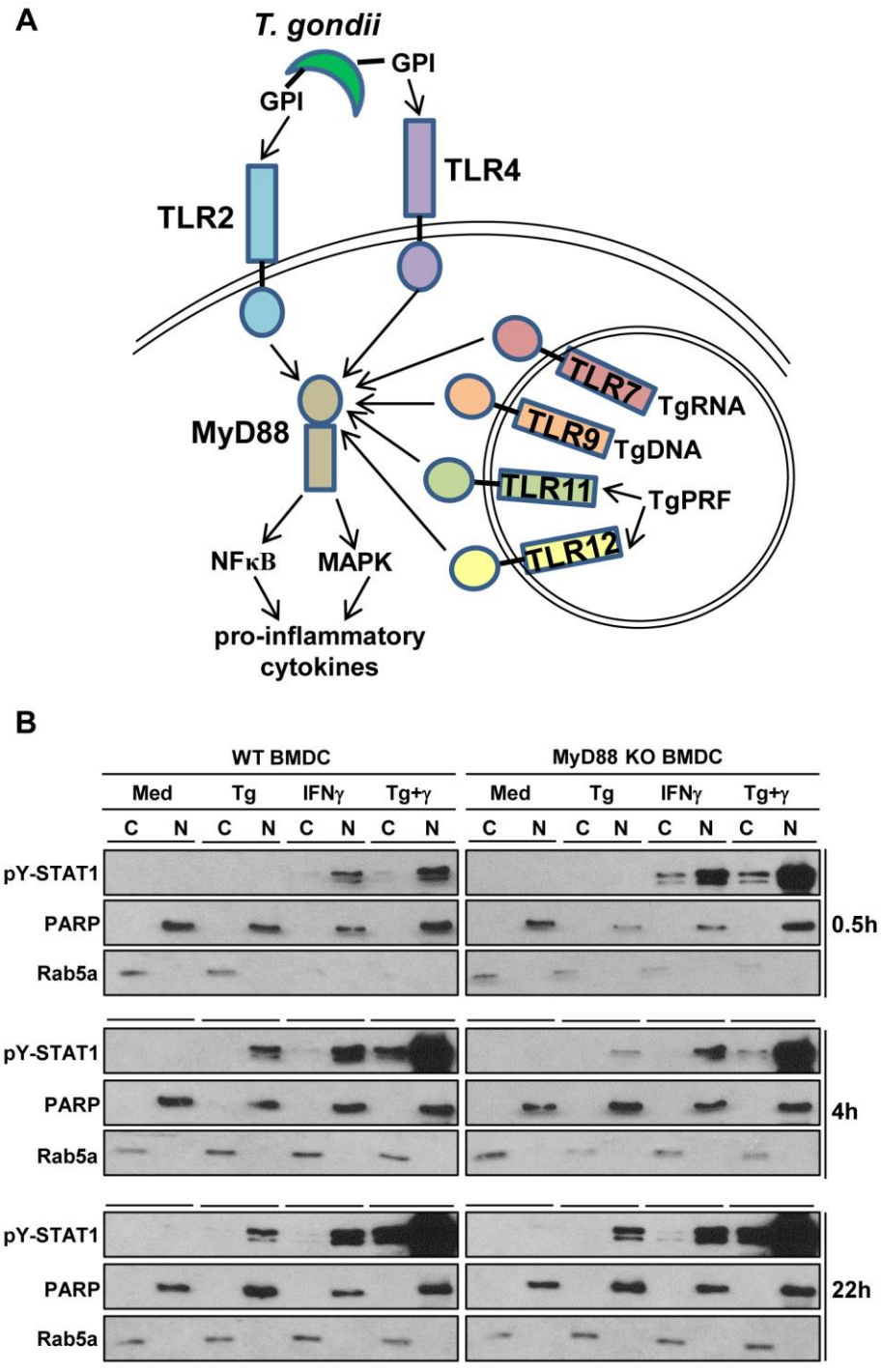
Not only were we seeking a mechanism for parasite-induced STAT1 phosphorylation, but we were also interested in understanding why synergistic levels of phospho-STAT1 were achieved in the combined presence of *Toxoplasma* plus IFNγ (50). In thinking about possible mechanisms for both, we investigated other host signaling pathways already known to be triggered by the

parasite. It is well-established that cross-talk that can occur between signaling pathways, such as between the toll-like receptor (TLR) and IFNγ-receptor pathways (66-69). Such signaling interactions could potentially lead indirectly to STAT1 phosphorylation in infected cells or even explain synergistic phospho-STAT1 levels when parasite and IFNγ were combined.

Toxoplasma is known to activate several TLR pathways through various parasite ligands (Figure 3.4A). In fact, MyD88, an important adaptor protein downstream of all TLRs except for TLR3 (57, 70), is critical for survival during in vivo *Toxoplasma* infection (71, 72). Glycosylphosphoinositol (GPI) anchors derived from *Toxoplasma* have been shown to activate TLRs 2 and 4; however, these TLRs are not thought to be critical to the immune response as mice deficient in those TLRs have a mild to no phenotype (73, 74). TLR11, which recognizes *Toxoplasma* profilin-like protein, is thought to play a role in dendritic cell sensing of the parasite; however, TLR11-deficient animals are only modestly affected in terms of survival compared to MyD88-deficient mice (75, 76). Recently, TLR12 was identified as a key sensor of *Toxoplasma* profilin, with TLR12-deficient mice demonstrating acute susceptibility to infection (77, 78). In addition, TLRs 7 and 9 have been recently shown to recognize parasite RNA and DNA, respectively (78). Although deficiency of these individual nucleic-acid sensing TLRs have no to modest impact on infection survival, it has become clear that the endosomal MyD88-coupled TLRs as group (TLRs 7, 9, 11, 12) act in concert to impart innate resistance to *Toxoplasma* (78, 79). To investigate whether TLR signaling could impact STAT1 phosphorylation, we compared infected wild-type and MyD88-deficient BMDC with IFNγ serving as a positive control. As expected, IFNγ-mediated STAT1 phosphorylation was unaffected by the absence of MyD88 (Figure 3.4B). When infected cells were compared, it was clear that MyD88-dependent

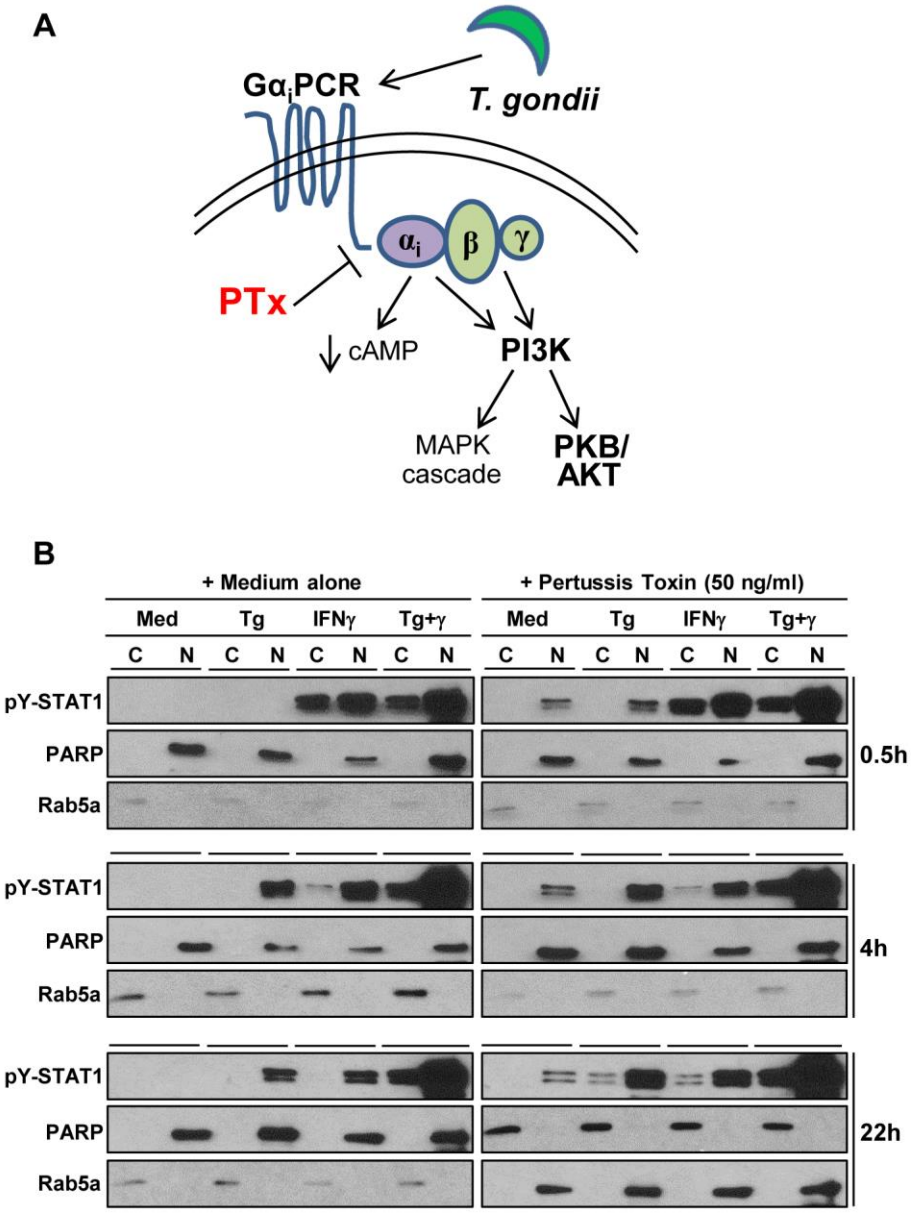
signaling was not required for parasite-mediated STAT1 phosphorylation at both early and late time points (Figure 3.4B). To investigate whether signaling pathway cross-talk could account for synergistic STAT1 phosphorylation, we compared wild-type and MyD88 knock-out BMDC after first pre-infecting with parasite, followed by IFNγ stimulation. As seen in Figure 3.4B, there was no appreciable difference in STAT1 phosphorylation levels between these groups at either early or late time points. Therefore, TLR signaling pathways dependent on MyD88 are not required for either STAT1 phosphorylation due to parasite infection alone or due to synergy with IFNγ.

Figure 3.4. *Toxoplasma*-mediated STAT1 phosphorylation and synergy with IFNγ do not require the TLR adaptor protein MyD88. (A) Schematic illustrating how *Toxoplasma* ligands activate various TLRs in a MyD88-dependent fashion both on the cell surface and within intracellular compartments. Activation of MyD88-dependent TLRs ultimately leads to activation of NFκB and MAP kinase signaling cascades, resulting in pro-inflammatory cytokine production. Tg, *Toxoplasma gondii*; TgPRF, *Toxoplasma* profilin; GPI, glycosylphosphatidylinositol-anchored proteins. (B) Wild-type (WT) BMDC were compared with MyD88 knock-out DC (KO) in terms of STAT1 tyrosine phosphorylation. DC were left in medium only (Med), treated with IFNγ (100 ng/ml), or infected with *Toxoplasma gondii* (Tg, RH strain) at a ratio of 3 parasites per cell for the specified time points. For some samples, BMDC were pre-infected with the parasite for 2 hours, followed by IFNγ stimulation for the time points indicated (Tg+γ). Cytoplasmic and nuclear extracts were prepared and subjected to immunoblot analysis for phospho-STAT1-Tyr701 (pY-STAT1). PARP and Rab5a served as nuclear and cytoplasmic loading controls, respectively. The experiment was performed twice with similar results.


A
T. gondii
GPI
GPI
TLR2
TLR4
TLR7
TgRNA
TLR9
TgDNA
TLR11
TgPRF
TLR12
MyD88
NFκB
MAPK
pro-inflammatory
cytokines
B
WT BMDC
MyD88 KO BMDC
Med
Tg
IFNγ
Tg+γ
C
N
pY-STAT1
PARP
Rab5a
0.5h
4h
22h

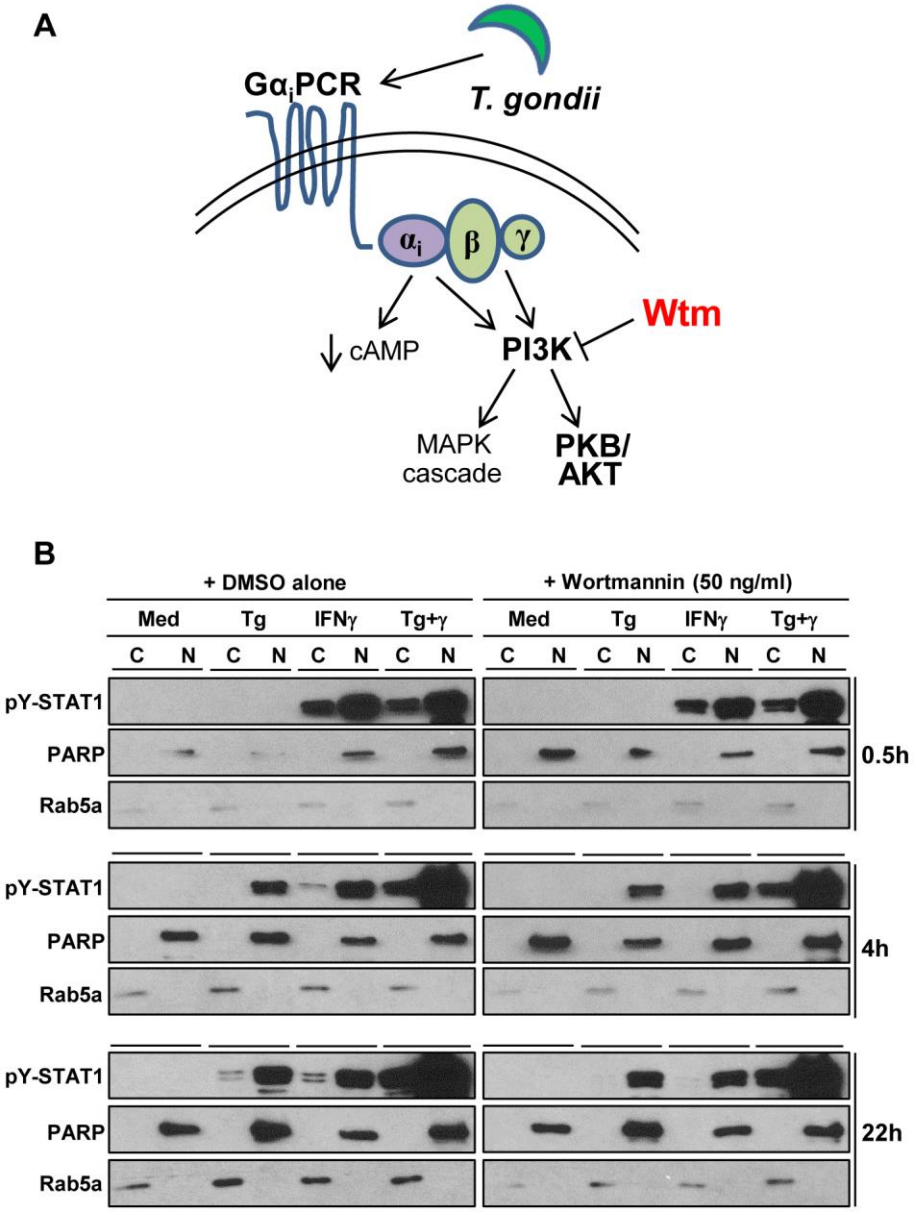
We next considered whether G protein coupled receptor (GPCR)-mediated signaling could account for the observed STAT1 phosphorylation response. Chemokine receptors are known to signal through G proteins with an inhibitory alpha subunit ($G\alpha_i$) (80). *Toxoplasma* has previously been shown to induce cysteine-cysteine chemokine receptor (CCR)-5-dependent interleukin-12 (IL12) production in dendritic cells (81) by secreting *Toxoplasma* cyclophilin-18 (TgCyc18) (82). TgCyc18 has also been shown to induce IL12 and tumor necrosis factor alpha (TNFα) in macrophages as well as NO production leading bradyzoite conversion, all in a CCR5-dependent manner (83). In addition, *Toxoplasma* is known to trigger phosphorylation of protein kinase B (PKB)/AKT in a non-CCR5 but G_iPCR-dependent manner (84). To explore whether G_iPCR signaling could play a role in STAT1 phosphorylation, we treated cells with pertussis toxin (PTx), which uncouples the G_i protein from the G_iPCR via ADP-ribosylation of the $G_i\alpha$ subunit (85) (Figure 3.5A). As expected, cells treated with IFNγ as a positive control were unaffected by a blockade of G_iPCR signaling (Figure 3.5B). Of note, when BMDC were treated with pertussis toxin, some basal STAT1 phosphorylation was observed in the medium alone control group at all time points examined (Figure 3.5B). PTx has been previously reported to induce STAT1 phosphorylation in brain endothelial cells, although the mechanism is unknown (86). Regardless, taking the basal STAT1 level into account, it is clear that the STAT1 phosphorylation response to parasite alone remains intact in the presence of PTx (Figure 3.5B). In addition, when cells are infected with *Toxoplasma* prior to IFNγ treatment, synergistic STAT1 phosphorylation persists in the presence of the toxin (Figure 3.5B). We conclude that parasite-mediated STAT1 tyrosine phosphorylation and synergy with IFNγ do not require G_iPCR-dependent signaling.

Figure 3.5. G_i protein-coupled receptors (G_iPCRS) are not required for *Toxoplasma*-induced STAT1 phosphorylation or synergy with IFNγ. (A) Schematic of a G_iPCR-dependent pathway triggered by *Toxoplasma*, with the site of action of pertussis toxin (PTx) indicated. The parasite is known to trigger AKT/PKB phosphorylation (pathway indicated in bold) in macrophages in a manner sensitive to PTx. (B) BMDC were pre-treated with pertussis toxin (PTx, 50 ng/ml) for 2 hours or incubated in medium only, prior to the addition of medium alone (Med), *Toxoplasma* (Tg, RH strain) at a ratio of 3 parasites per cell, or IFNγ (100 ng/ml) in the continued presence of the drug for the time points indicated. For some samples, BMDC were pre-infected with the parasite for 2 hours, followed by IFNγ stimulation for the specified time points (Tg+γ). Cytoplasmic and nuclear extracts were prepared and subjected to immunoblot analysis for phospho-STAT1-Tyr701 (pY-STAT1). PARP and Rab5a served as nuclear and cytoplasmic loading controls, respectively. The experiment was performed twice with similar results.


A
GαiPCR
T. gondii
αi
β
γ
PTx
cAMP
PI3K
MAPK cascade
PKB/ AKT
B
+ Medium alone
+ Pertussis Toxin (50 ng/ml)
Med
Tg
IFNγ
Tg+γ
C
N
pY-STAT1
PARP
Rab5a
0.5h
4h
22h

Finally, we considered whether a phosphoinositide 3-kinase (PI3K)-dependent signaling pathway could be involved. As illustrated in Figure 3.6A, *Toxoplasma* is known to trigger PKB/AKT phosphorylation in dependence not only on an unknown G_iPCR, but also on PI3K (84). In addition, the parasite can trigger production of a variety of chemokines in macrophages in a PI3K-dependent manner (87). A requirement for PI3K signaling can be explored by treating cells with the specific and potent inhibitor, wortmannin (88) (Figure 3.6A). We treated BMDC with wortmannin or with the solvent DMSO alone prior to infecting cells for a STAT1 phosphorylation time course. As anticipated, there was no requirement for PI3K in IFNγ-induced STAT1 tyrosine phosphorylation (Figure 3.6B). When *Toxoplasma*-infected cells were compared, STAT1 phosphorylation was unaffected by wortmannin treatment (Figure 3.6B). Finally, we observed no decrease in synergistic STAT1 phosphorylation when infected cells were treated with IFNγ in the presence of inhibitor (Figure 3.6B). In summary, parasite-triggered STAT1 tyrosine phosphorylation does not require PI3K, MyD88 or G_iPCRs nor does the observed synergy with IFNγ rely on cross-talk with such pathways.

Figure 3.6. Phosphoinositide 3-kinase (PI3K) activity is not required for the observed impacts of *Toxoplasma* on STAT1 phosphorylation. (A) Diagram of a PI3K-dependent pathway initiated by *Toxoplasma*, with the site of action of the selective PI3K inhibitor, wortmannin (Wtm), indicated. The parasite is known to cause AKT/PKB phosphorylation in macrophages in a wortmannin-sensitive manner (pathway indicated in bold). (B) BMDC were pre-treated with wortmannin (Wtm, 50 ng/ml) or an equivalent concentration of DMSO only (solvent control) for two hours prior to addition of medium alone (Med), *Toxoplasma* (Tg, RH strain) at a ratio of 3 parasites per cell, or IFNγ (100 ng/ml) in the continued presence of the drug for the time points indicated. For some samples, BMDC were first pre-infected with the parasite for 2 hours, followed by IFNγ stimulation for the time points specified (Tg+γ). Cytoplasmic and nuclear extracts were prepared and subjected to immunoblot analysis for phospho-STAT1-Tyr701. PARP and Rab5a served as nuclear and cytoplasmic loading controls, respectively. The experiment was performed twice with similar results.


A
GαiPCR
T. gondii
αi
β
γ
Wtm
cAMP
PI3K
MAPK cascade
PKB/ AKT
B
+ DMSO alone
+ Wortmannin (50 ng/ml)
Med
Tg
IFNγ
Tg+γ
C
N
pY-STAT1
PARP
Rab5a
0.5h
4h
22h

Parasite-mediated inhibition of IFNγ-induced SOCS1 may drive elevated phospho-STAT1 levels

One possible mechanism to explain the high levels of phosphorylated STAT1 in infected cells treated with IFNγ is signaling pathway synergy. However, at least in the case of MyD88, G_iPCRs, and PI3K, that did not appear to be the case (Figures 3.4-3.6). Alternatively, *Toxoplasma* could be interfering with the regulation of the IFNγ-signaling response, preventing the normal dephosphorylation of STAT1. A key regulator protein induced by IFNγ is suppressor of cytokine signaling 1 (SOCS1), a phosphatase that decreases STAT tyrosine phosphorylation indirectly by interfering with the upstream Janus kinase (JAK) activity (89). To determine what involvement SOCS1 may play in dendritic cells, we examined *Socs1* expression by qPCR following IFNγ stimulation or parasite infection. Given that a strain-dependent induction of *Socs1* by *Toxoplasma* had been previously reported in other cell types (15, 47, 90), we initially compared a virulent type I strain with a less virulent type II strain. The type II strain did not induce significant *Socs1* expression, whereas the type I strain triggered a significant 10-12 fold increase (Figure 3.7A). Furthermore, the ability of the type I strain to induce *Socs1* was confirmed to be dependent on the rhoptry kinase ROP16, as parasites of the deleted strain ($\Delta 16_I$) could not induce comparable expression (Figure 3.7C). Parasite induction of *Socs1* was modest in comparison to IFNγ, however, which triggered ~60-90-fold increases in *Socs1* expression (Figure 3.7B, D). However, when infected cells were subsequently treated with IFNγ, *Socs1* levels were significantly reduced in a strain-independent manner ~3 fold when compared to IFNγ alone (Figure 3.7B, D). In summary, although *Toxoplasma* induces modest ROP16-dependent *Socs1* expression, IFNγ-mediated upregulation of that key JAK-STAT1 signaling regulator is blocked by the parasite. This suggests a mechanism for the observed high levels of phospho-

STAT1, whereby the parasite blocks the normal IFNγ-induced negative feedback loop mediated in part by SOCS1.

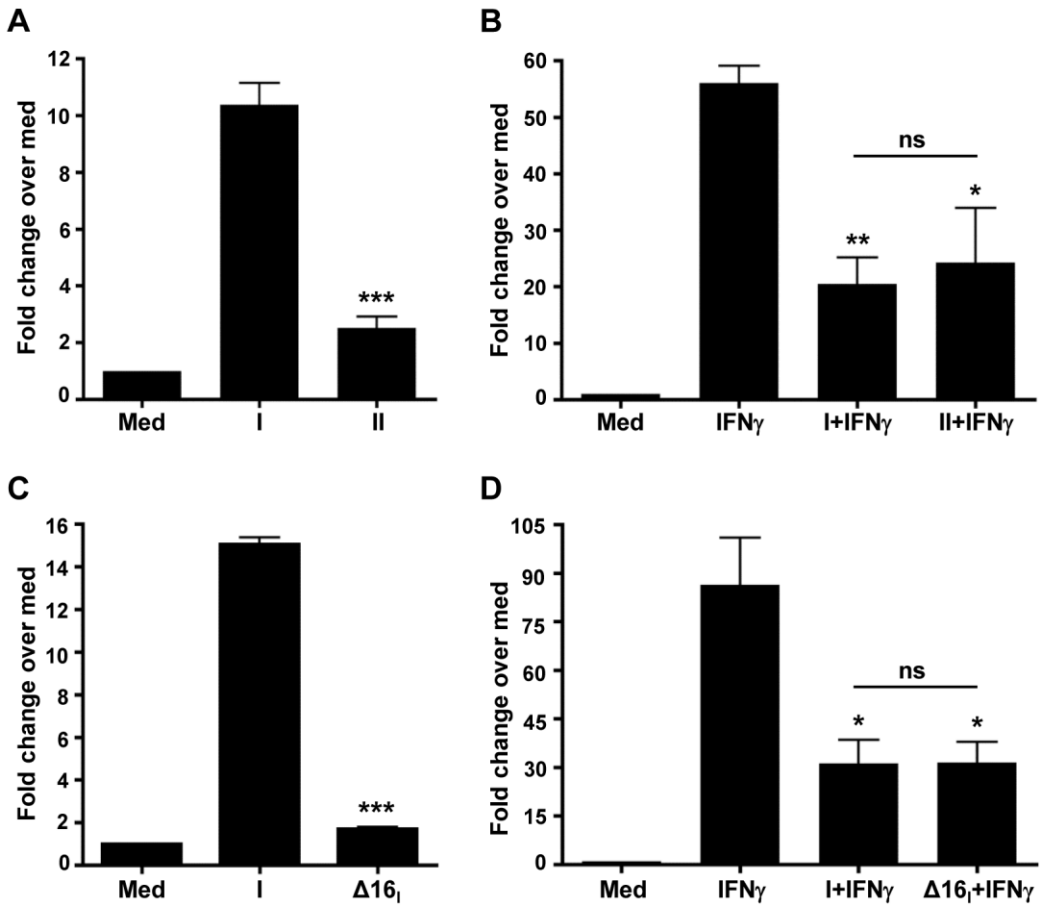


Figure 3.7. Despite modest ROP16-dependent induction of *Socs1* by *Toxoplasma* alone, the parasite blocks IFNγ-induced *Socs1* expression independently of strain type. (A, C) BMDC were treated with medium alone (Med), IFNγ (100 ng/ml), or infected with various *Toxoplasma* strains (Type I – RH (I), Type II – PTG (II), or ΔROP16 on an RH background ($\Delta16_I$)) at a ratio of 3 parasites per cell for 2 hours. Total RNA was prepared and reverse-transcribed to cDNA prior to qPCR analysis of the IFNγ-responsive *Socs1* gene. Fold change in gene expression is plotted relative to medium alone samples (set to 1). Samples were normalized to the house-keeping gene GAPDH. (B, D) Cells were treated, prepared, and analyzed as in (A, C), with the exception that for some samples, BMDC were first pre-infected with *Toxoplasma* for 2 hours prior to addition of IFNγ for another 2 hours (I+IFNγ, II+IFNγ, $\Delta16_I$+IFNγ). Experiments were performed three times with similar results. * = $p < 0.05$; ** = $p < 0.01$, *** = $p < 0.001$ when compared to IFNγ treatment alone. Ns, not significant.

Discussion

From our studies and others, it has become clear that blockade of IFNγ/STAT1-dependent transcriptional responses is a key component of immune evasion by *Toxoplasma*. By blocking such responses, parasites can successfully maintain the integrity of the parasitophorous vacuole against antimicrobial effector mechanisms. This borrowed time in turn allows the parasite to eventually convert to the persistent, life-long state of the bradyzoite, secure within a tissue cyst. Yet, paradoxically, the parasite is able to induce some degree of STAT1 phosphorylation on its own, in the absence of IFNγ. Indeed, parasites synergize with IFN-γ to induce high amounts of STAT1 phosphorylation. In this study, we ruled out mechanisms for STAT1 transcriptional inhibition, phosphorylation and synergy, while implicating parasite-mediated inhibition of IFNγ-driven *Socs1* expression in the mechanism of phospho-STAT1 accumulation.

The precise mechanism of how *Toxoplasma* inhibits IFNγ/STAT1-dependent gene transcription remains unclear. We speculated that STAT3 could play a role in the inhibition of STAT1 activity, given that the parasite induces STAT3 phosphorylation directly via ROP16, which has profound effects on inhibition of pro-inflammatory cytokine production (12, 14, 15, 91). In addition, there is some evidence to suggest that STAT3 can antagonize STAT1 activity (61, 92). However, inhibition of IFNγ-induced gene expression occurs independently of the parasite strain type. Thus, the rhoptry kinase ROP16, which is the major inducer of STAT3 phosphorylation in infected cells of the type I or type III clonal lineages, is unlikely to contribute in a major way to inhibition of STAT1 activity, a conclusion supported by microarray data performed in the RAW264.7 cell line (23). However, STAT3 involvement could not be completely ruled out as some phosphorylation also occurs independently of ROP16 in infected cells. We found that

STAT3 could associate with STAT1 in infected cells. However, STAT3 was not detected as part of an aberrant STAT1-containing GAS-binding complex, a complex speculated to play a role in STAT1 inhibition. Whether STAT3 could contribute to STAT1 inhibition by some other mechanism requires further study.

Another intriguing possibility is that the parasite induces transcriptionally active STAT1/STAT3 heterodimers, which could regulate a distinct subset of genes from IFNγ. The absence of STAT3 binding by EMSA could be explained by STAT1/STAT3 heterodimers recognizing a consensus sequence distinct from GAS. This hypothesis could explain the role of STAT1 phosphorylation in response to parasite alone while also allowing for inhibition of IFNγ transcription by another mechanism. On the other hand, it is possible that what we detected through co-immunoprecipitation assay was not a STAT1/STAT3 heterodimer, but rather an indirect association through an intermediate. This interpretation could explain the STAT1/3 association in response to IFNγ treatment, as IFNγ can induce phosphorylation of both proteins through common JAK molecules (62). Furthermore, the enhanced association of STAT1 and 3 in the combined presence of parasite and IFNγ could occur due to impairment of SOCS expression, allowing IFNγ-triggered JAKs to continuously induce STAT1/3 phosphorylation. The functional relevance of the parasite-induced STAT1/STAT3 association will be a future area of interest to pursue.

STAT1 phosphorylation in *Toxoplasma*-infected cells without IFNγ stimulation has recently been characterized by us and Saeij and colleagues (23, 50). The mechanism by which this occurs remains unknown. We previously ruled out involvement of the rhoptry kinase ROP16, known to

phosphorylate host STAT3 and STAT6 (50). Here, we rule out involvement of additional parasite rhoptry proteins, ROP18 and ROP21. From our previous work, we would not necessarily have expected a polymorphic rhoptry kinase to play a role, as STAT1 phosphorylation occurs in infected dendritic cells regardless of parasite strain type (50). This complicates elucidation of the mechanism of STAT1 phosphorylation, as most of the key secreted parasite effector proteins have been identified and characterized through pair-wise genetic crosses between strain types, with subsequent analysis for genome loci that account for virulence differences (reviewed in (93)). It seems more likely that the parasite molecule accounting for STAT1 phosphorylation is not involved in determining such virulence differences, but rather is a common feature among the three main strain types. Indeed, the dominant *Toxoplasma* clonal lineages only differ from one another by ~1-2% in terms of genome sequence (10), so to have a strain-independent response would not be unexpected.

We hypothesize that a parasite dense granule protein may be responsible, as the delayed kinetics of STAT1 phosphorylation compared to STAT3 phosphorylation would suggest (50). Dense granule protein exocytosis does not generally occur until the parasite has completed parasitophorous vacuole formation, continuing throughout the intracellular infection (94, 95). In addition, the similar delayed kinetics of NF-κB activation due to activity of the dense granule protein GRA-15 lend further support to this idea (22). Although GRA-15 appears to be active in type II strains only, another dense granule protein expressed independent of strain type, such as GRA-7 (96), would be a candidate effector. At this point it remains unclear whether parasite-activated STAT1 serves a purpose during infection distinct from that of IFNγ, or whether STAT1

is activated in a bystander fashion at low levels, rendered irrelevant due to potent downstream transcriptional repression.

Toxoplasma blocks IFNγ/STAT1-dependent transcription, yet does not negatively impact STAT1 phosphorylation in response to IFNγ in the majority of cell types examined. In fact, in infected dendritic cells we observed synergistic levels of STAT1 phosphorylation. We speculated that this could be a result of signaling pathway cross-talk. We found that other major signaling pathways triggered by the parasite did not play a role in either STAT1 tyrosine phosphorylation by the parasite alone, or in the observed synergy with IFNγ. Although MyD88 does not appear to play role in terms of TLR-mediated pathways, we cannot rule out the possible involvement of other TLR adaptors, namely TRIF/TICAM-1. In addition, we ruled out G_iPCR and PI3K pathway involvement in STAT1 activation and synergy with IFNγ through inhibitor experiments. It remains possible that cross-talk with another untested signaling pathway initiated by the parasite could contribute to elevated STAT1 levels.

We also considered whether the parasite could be blocking normal regulation of the IFNγ/STAT1 pathway. IFNγ-triggered STAT1 phosphorylation occurs rapidly and is quickly tempered by the activity of several phosphatases (97). We confirmed that *Toxoplasma* can inhibit IFNγ-triggered expression of a key phosphatase, *Socs1*, in dendritic cells and that this inhibition occurs independently of parasite strain type. This result corroborates microarray data collected in other cell types that noted inhibition of IFNγ-driven *Socs1* expression (48, 49). *Socs1* inactivates a Janus kinase downstream of the IFNγ receptor, thereby preventing IFNγ from triggering additional STAT1 tyrosine phosphorylation (98). By preventing *Socs1* expression,

that level of regulation is lost, allowing the Janus kinase to activate STAT1 continuously in the presence of IFNγ. Thus, inhibition of this key phosphatase is likely to explain how synergistic levels of phospho-STAT1 accumulate over time. To further corroborate this finding, it would be of interest to over-express SOCS1 and determine whether the synergistic effects of *T. gondii* on phospho-STAT1 levels are abrogated. Other phosphatases known to regulate IFNγ/JAK/STAT1 signaling such as the protein tyrosine phosphatases SHP-1 and SHP-2 as well as the nuclear phosphatase TC-45 could also potentially be targeted and contribute to elevated phospho-STAT1 levels (97). However, because these phosphatases were not identified among the IFNγ-inducible genes down-regulated by *Toxoplasma gondii* (48, 49), inhibition of *Socs1* expression remains the most likely explanation for the synergistic phospho-STAT1 levels observed.

Interestingly, even though *Toxoplasma* blocks IFNγ-triggered *Socs1* expression, the parasite itself induces a modest amount of *Socs1* in a ROP16-dependent manner, in line with findings in other cell types (15, 47, 90). It is unclear at this time what role parasite-induced, strain-dependent *Socs1* may play, but it does not appear to affect STAT1 phosphorylation insofar as the latter is induced in a strain-independent manner. However, SOCS1 has been shown to negatively regulate TLR signaling (99). It is conceivable that the parasite induces SOCS family proteins as part of an immune evasion mechanism to counteract TLR or other *Toxoplasma*-sensing pathways while blocking IFNγ-mediated gene expression in another manner. Further work is needed to identify targets and the functional outcome of parasite-induced SOCS1 in infected cells. In summary, we have identified inhibition of *Socs1* expression as a likely mechanism for synergistic STAT1 phosphorylation in infected cells treated with IFNγ, while also ruling out

some key potential mechanisms for STAT1 phosphorylation and inhibition of STAT1 activity by *Toxoplasma*.

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CHAPTER 4

Discussion

Summary of findings

Interferons are critical for host control of a wide variety of pathogens, including viruses, intracellular bacteria, and protozoa. STAT1 is a key downstream signaling molecule activated by both type I (IFNα/β) and type II (IFNγ) interferons. In fact, STAT1 loss of function mutations in humans are linked to increased susceptibility to mycobacterial and viral infections (1-3). Inhibition of interferon and/or STAT1-dependent activity is thus a key strategy employed by many intracellular pathogens to evade the host immune response. Both type I and II interferons have anti-viral properties, although type I interferons are particularly important as they are directly induced in virus-infected cells (4). Viruses have evolved multiple strategies for evasion of IFNα/β responses, including those that target STAT1 for inactivation (4). IFNγ and STAT1 are essential in the control of various intracellular bacteria and protozoa. Intracellular bacteria such as *Mycobacterium tuberculosis* and enteropathogenic *Escherichia coli* can interfere with IFNγ-dependent transcriptional responses/chromatin remodeling (5, 6) or STAT1 activation (7, 8), respectively, to promote survival. In addition, the protozoan *Leishmania* has been shown to promote STAT1 degradation as a means of regulating the IFNγ response (9). Related to the topic of this thesis, *Toxoplasma* is another example of a protozoan that hijacks the IFNγ/STAT1 pathway to facilitate survival. The content of this thesis confirms the importance of blocking this pathway in a key immune cell type, the dendritic cell. In addition, I further investigate the mechanisms by which *Toxoplasma* impairs IFNγ-mediated STAT1 transcriptional responses while paradoxically triggering STAT1 phosphorylation upon infection alone.

The data in Chapter 2 surprisingly reveal that infection of dendritic cells with *Toxoplasma* in the absence of IFNγ is enough to trigger sustained STAT1 phosphorylation and nuclear

translocation. The parasite initiates both tyrosine and serine phosphorylation at key residues of STAT1. Phosphorylation can be weakly detected as early as 30 minutes post-infection, but does not achieve peak levels until several hours later. This occurs in contrast to the cytokine IFNγ, which induces rapid and robust STAT1 phosphorylation within the first 30 minutes that subsequently declines over time. Furthermore, when DCs are infected with *Toxoplasma* prior to IFNγ treatment, synergistic levels of phosphorylated STAT1 are obtained. Activation of STAT1 occurs independently of parasite strain type when comparing representatives of the three main clonal lineages. In agreement with this, the rhoptry kinase ROP16 does not play a role, despite the ability of this parasite kinase to induce STAT3/6 phosphorylation. Further investigation reveals that live parasites are required for STAT1 phosphorylation. Furthermore, the parasites must actively invade cells rather than just attaching to the cell surface as experiments with cytochalasin D treatment confirm. Use of a replication-deficient strain shows that parasite replication is not required to maintain STAT1 phosphorylation. Transfer of supernatant from infected cells to uninfected cells confirms that a soluble mediator is not responsible for the observed response. In addition, STAT1 phosphorylation is confined to infected cells, indicating that surface contact between infected and uninfected cells is also not a possible mechanism.

I next looked at the ability of parasite-induced STAT1 to bind to DNA. Parasite alone is able to induce modest STAT1 binding to GAS elements in a transcription factor binding ELISA, although chemiluminescent EMSA does not appear to be sensitive enough to detect this. However, cells that were pre-infected with the parasite and subsequently treated with IFNγ demonstrate synergistic levels of STAT1 GAS binding by EMSA. In addition, an aberrant STAT1-containing complex is identified in infected cells in addition to the classic gamma-

activated factor (STAT1 homodimer) induced by IFNγ alone. However, despite evidence of STAT1 binding to consensus sequences, ChIP assays reveal that the parasite does not induce STAT1 binding to the native *Irf1* promoter. In addition, the parasite blocks IFNγ-induced STAT1 promoter recruitment. In agreement with this, transcription of IFNγ/STAT1 responsive genes including *Irf1* and the p47 GTPases are also blocked by *Toxoplasma*. Therefore, although the parasite can induce STAT1 phosphorylation in synergy with IFNγ in DCs, transcription of IFNγ-induced STAT1 responsive genes remains blocked.

In Chapter 3, I further define the mechanisms by which *Toxoplasma* inhibits IFNγ-induced STAT1 transcriptional activity, induces STAT1 phosphorylation, and synergizes with IFNγ in terms of STAT1 phosphorylation. I show that inhibition of STAT1 transcriptional activity by the parasite occurs independently of parasite strain type. In agreement with this, ROP16-deleted parasites are equally able to inhibit STAT1 transcription compared to wild-type, eliminating ROP16-dependent STAT3 activation as a contributor to STAT1 inhibition. Furthermore, I show that STAT3 does not contribute to aberrant STAT1 complex formation by EMSA through a supershift experiment, making STAT3 a less likely culprit for STAT1 inhibition. However, STAT1/STAT3 interactions are detected in infected cells, raising the possibility that parasite-induced STAT3 may participate in regulating a different subset of genes via STAT1/3 heterodimers. In terms of STAT1 phosphorylation by the parasite, it is clear that the rhoptry molecules ROP18 and ROP21 do not contribute, as infections with mutant strains demonstrate. In addition, G_iPCR-, PI3K-, and MyD88-dependent pathways triggered by the parasite upon invasion also do not contribute to either parasite-induced STAT1 phosphorylation or the synergy observed with the IFNγ pathway. I also investigated whether inhibition of a key regulator of

IFNγ-dependent JAK/STAT1 signaling, SOCS1, could explain the synergistic levels of phosphorylated STAT1 observed in infected cells treated with IFNγ. It is clear that the parasite downregulates *Socs1* expression induced by IFNγ, strongly implicating *Socs1* inhibition in the mechanism for enhanced STAT1 phosphorylation. Interestingly, the parasite alone also induces a modest level of strain-dependent *Socs1* expression, in dependence on the rhoptry kinase ROP16. Parasite-induced *Socs1* cannot account for STAT1 inhibition, given that transcriptional inhibition occurs independently of strain type, but could potentially play a role in the strain-dependent inactivation of TLR-induced pro-inflammatory cytokine production. This chapter serves to rule out several potential key players in the mechanism of STAT1 phosphorylation and transcriptional inhibition, while implicating inhibition of SOCS1 as the cause of synergistic phosphorylated STAT1 levels.

Future directions and unanswered questions

It is clear from prior *in vivo* studies that STAT1, in addition to IFNγ, is essential in host resistance to *Toxoplasma* infection (10, 11). Despite the parasite's efforts at blocking IFNγ/STAT1-dependent signaling in infected cells, uninfected cells of the innate immune system are capable of responding to the cytokine, ready to eliminate the parasite upon future encounter. It is unclear at this time which cell types are essential for STAT1-mediated resistance *in vivo*. Dendritic cells are considered the key IL12 producers in vivo during *Toxoplasma* infection and help initiate adaptive immune responses, but macrophages also possess a potent arsenal of STAT1-dependent antimicrobial effector mechanisms (12). Conditional ablation of STAT1 in dendritic cell versus macrophage subsets using Cre/Lox technology followed by infection with *Toxoplasma* would help to identify the relative importance of STAT1 signaling in innate cell

types. A precedent for this has already been established during *Listeria* infection, where macrophage STAT1 is considered protective while DC STAT1 has no effect on host survival (13). However, DC STAT1 does contribute positively to secondary responses in vaccinated animals upon challenge. Similar studies could be performed with *Toxoplasma* to determine relative impact on survival of macrophage versus dendritic cell STAT1 during acute infection and as well as during secondary responses in a vaccination model.

The data presented in Chapter 2 demonstrate that *Toxoplasma* can induce STAT1 phosphorylation in the absence of additional IFNγ. In agreement with my work, another recent study had a similar finding in infected human fibroblasts, although those authors found ROP16 to be required for STAT1 phosphorylation (14). However, this is not the case for murine dendritic cells, leaving the mechanism yet to be defined. The data support a direct effect of *Toxoplasma* on host cell signaling, rather than induction of a soluble mediator. In addition, the delayed kinetics of STAT1 phosphorylation is comparable to the kinetics of NF-κB activation induced by the parasite dense granule protein GRA15 (15). In agreement with this, induction of STAT1 phosphorylation is inhibited by cytochalasin D treatment, which prevents host cell invasion as well as dense granule protein release by the parasite (16). Taken together, the data implicate a parasite-derived effector protein in STAT1 phosphorylation that is likely derived from the dense granules. Unlike GRA15 activity, STAT1 phosphorylation by the parasite occurs in a strain-independent manner. Future work to identify a strain-independent GRA protein may serve to identify the parasite molecule responsible. To this end, GRA7 is expressed by all three strain types and may be a viable candidate to explore through use of deletion mutants (17). Additional GRA proteins known to associate with the parasitophorous vacuole membrane

include GRA3, 5, 8, and 10 (18). Deletion mutants of these parasites could be acquired/constructed and analyzed for ability to induce STAT1 phosphorylation. It may also be possible to acquire dense granule protein fractions from the different parasite strains to determine if dense granule protein is indeed involved.

The question remains as to why *Toxoplasma* induces STAT1 phosphorylation in the first place. Saeij and colleagues suggest that STAT1 phosphorylated in response to *Toxoplasma* infection is transcriptionally inactive, given that IFNγ-responsive genes are transcriptionally repressed. Furthermore, they suggest that ROP16 may phosphorylate STAT1 less efficiently compared to STATs3 and 6, with STAT1 being an accidental target (14). My data in murine dendritic cells does not rule out the possibility of bystander STAT1 activation by some parasite kinase, but it is clear that ROP16 does not play a role as STAT1 phosphorylation occurs independently of this molecule and parasite strain type in general. However, it is also possible that *Toxoplasma*-induced STAT1 may modulate transcription of a subset of genes distinct from the antimicrobial genes activated by IFNγ. The mechanism by which this might occur is unclear, but could involve formation of complexes with other transcription factors at different gene promoters. To investigate this possibility further, it would be of interest to compare *Toxoplasma*-infected STAT1 knock-out DCs with infected wild-type counterparts and perform microarray analysis to determine which host genes, if any, are positively or negatively regulated by the parasite in a STAT1-dependent manner. It is possible that parasite-controlled STAT1 activation plays a role in modulation of the host immune response or contributes to parasite survival through impacts on other pathways.

Data from Chapters 2 and 3 address inhibition of IFNγ-mediated STAT1 transcriptional activity by *Toxoplasma*. This work is the first to demonstrate inhibition of IFNγ responses by the parasite in the dendritic cell, an immune cell type that is critical during *in vivo* infection. Further work is required to identify what parasite molecule(s) carry out STAT1 inhibition, as well as the mechanism by which this occurs. Work performed in bone marrow-derived macrophages demonstrates that *Toxoplasma* can impair transcription-permissive histone acetylation as well as recruitment of the BRG1 component of a chromatin remodeling complex to the CIITA promoter (19). However, BRG1 is not required for IFNγ-induced remodeling of the *Irf1* promoter (20), suggesting that BRG1 may not the direct target of the parasite.

Interestingly, the parasite causes formation of aberrant STAT1-containing complexes that retain the ability to bind GAS oligonucleotides by EMSA, but not the native promoters of IFNγ-responsive genes in ChIP assays. Attempts to identify contributors to this complex by myself (Chapter 3) and others (19) have ruled out involvement of host STAT3, STAT2, and IRF9 but have so far failed in identifying the specific host or parasite proteins involved. It is tempting to speculate that the parasite secretes a protein upon invasion that binds to STAT1 and could account for the aberrant STAT1 complex formation (Figure 4.1). This interaction may allow binding to DNA oligonucleotides but could still impair association with key chromatin modifiers/remodelers such as BRG1 that are needed to establish stable native promoter binding and transcription. This would not be without precedent as an adenoviral protein, E1A, has been shown to inhibit STAT1 activity through direct interaction with STAT1, independent of the ability of E1A to compete with STAT1 for binding to the histone acetylase p300/CBP (21). *Mycobacterium tuberculosis* can also impair chromatin remodeling at the IFNγ-responsive

CIITA promoter in dependence upon a 19 kDa lipoprotein, although the exact mechanism by which this occurs is still unclear (6). It has also been shown that impaired chromatin remodeling occurs at the TNFα promoter in *Toxoplasma*-infected macrophages (22). However, the mechanism behind inhibition of LPS-induced IL12 and TNFα production is different from STAT1 inhibition, as the former is strain/ROP16-dependent (23) while the latter is not. For STAT1 inhibition, future studies should focus on identifying the additional components in the aberrant STAT1 complex. Attempts to immunoprecipitate native STAT1 in primary cells directly or indirectly via GAS oligonucleotide probes have so far failed to yield candidates. To improve sensitivity, it may be useful to transfect or transduce STAT1-deficient cells with a tagged STAT1 construct prior to infecting them and performing a pull-down assay using beads directly conjugated to antibodies against the tag. Upon elution, the "prey" including STAT1 and any associated molecules could be separated by gel electrophoresis. Candidate protein bands could then be submitted for mass spectrometric analysis. If a candidate *Toxoplasma* protein is identified, a mutant parasite could be generated to confirm if that protein plays a role in STAT1 transcriptional inhibition.

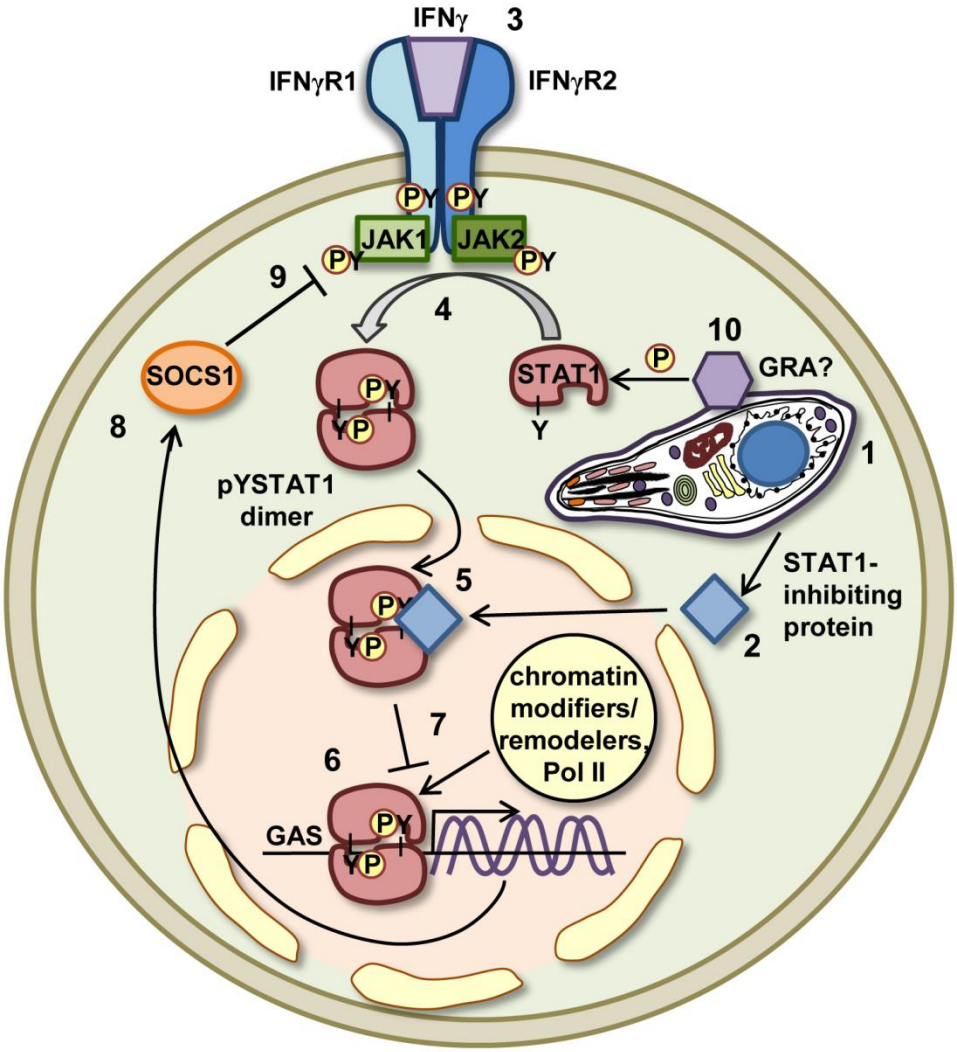

Figure 4.1. Proposed model for *Toxoplasma*-induced STAT1 phosphorylation and inhibition of IFNγ-driven STAT1 transcription. *Toxoplasma* establishes intracellular infection within the parasitophorous vacuole (1). The parasite may then secrete a protein capable of binding to STAT1 (2). At this point, the cell may come into contact with the cytokine IFNγ, which initiates a JAK/STAT1 signaling cascade upon binding to its receptor subunits (3). Activated JAKs phosphorylate STAT1 (4), forming dimers that translocate to the nucleus (5). However, the *Toxoplasma* STAT1-binding protein may then interact with STAT1 in the nucleus, preventing stable binding to native promoters of responsive genes (6) by impairing interactions with histone modifying and chromatin remodeling enzymes (7). This prevents transcription of various IFNγ-responsive genes including the negative regulator of IFNγ signaling, SOCS1 (8). As a result, SOCS1 cannot impair JAK activation as it normally would (9), resulting in accumulation of phospho-STAT1 in infected cells. In addition, another unknown *Toxoplasma* protein speculated to be a dense granule protein (GRA) may also trigger STAT1 phosphorylation, the function of which remains unknown.

It is also apparent from the data in Chapter 3 that *Toxoplasma* can significantly inhibit IFNγ-induced *Socs1* expression in DCs. This is consistent with microarray data from infected human fibroblasts and murine macrophages treated with IFNγ (19, 24). Given that SOCS1 normally downregulates IFNγ signaling by inactivating JAK activity, this could explain why high levels of phosphorylated STAT1 accumulate in infected cells treated with IFNγ. Further work to confirm this correlation would entail overexpression of SOCS1 in infected cells treated with IFNγ to see if the high level of phosphorylated STAT1 is subsequently lost. Despite this increased level of phosphorylated STAT1, *Toxoplasma* is still able to mediate inhibition of IFNγ-driven STAT1 transcriptional responses, rendering the STAT1 non-functional.

Paradoxically, *Toxoplasma* alone is also able to induce modest *Socs1* expression in a ROP16-dependent manner, as seen by us and others (25-27). However, SOCS1 does not appear to contribute to parasite-mediated inhibition of IFNγ signaling, given that STAT1 inhibition occurs independently of strain type. Another possibility is that parasite-induced SOCS1 contributes to inhibition of TLR signaling by the parasite. One study suggests that SOCS1 can regulate stability of the NF-κB subunit p65/RelA (28) while another indicates that SOCS1 may target IRAK1 (29). Strong evidence also exists for SOCS1 targeting of Mal/Tirap, an adaptor protein for TLRs 2 and 4. Mal/Tirap is phosphorylated on a tyrosine residue by Bruton's tyrosine kinase (BTK) which facilitates interaction of Mal with SOCS1 (30). SOCS1 subsequently mediates ubiquitination and proteasomal degradation of Mal (30). TRAF6 can no longer interact with Mal and this impairs subsequent serine phosphorylation of NF-κB that is required for transcriptional activity. Under these conditions, initial NFκB activation is unaffected (31, 32).

It is known that *Toxoplasma* inhibits LPS-induced IL12 and TNFα production in a ROP16-dependent manner, involving STAT3 activation (23). However, it is unclear how STAT3 exerts this effect. STAT3 can, however, induce expression of *Socs1*, at least in terms of IL10 signaling (33). Given the known effect STAT3 can have on *Socs1* expression, I propose that ROP16-activated STAT3 induces *Socs1* expression. This in turn could negatively regulate certain TLR pathways, thereby contributing to inhibition of proinflammatory cytokine production (Figure 4.2). This could explain how the parasite impairs NF-κB binding to the TNFα promoter and transcription (22) and/or contribute to inhibition of TLR-induced MAPK activation (34).

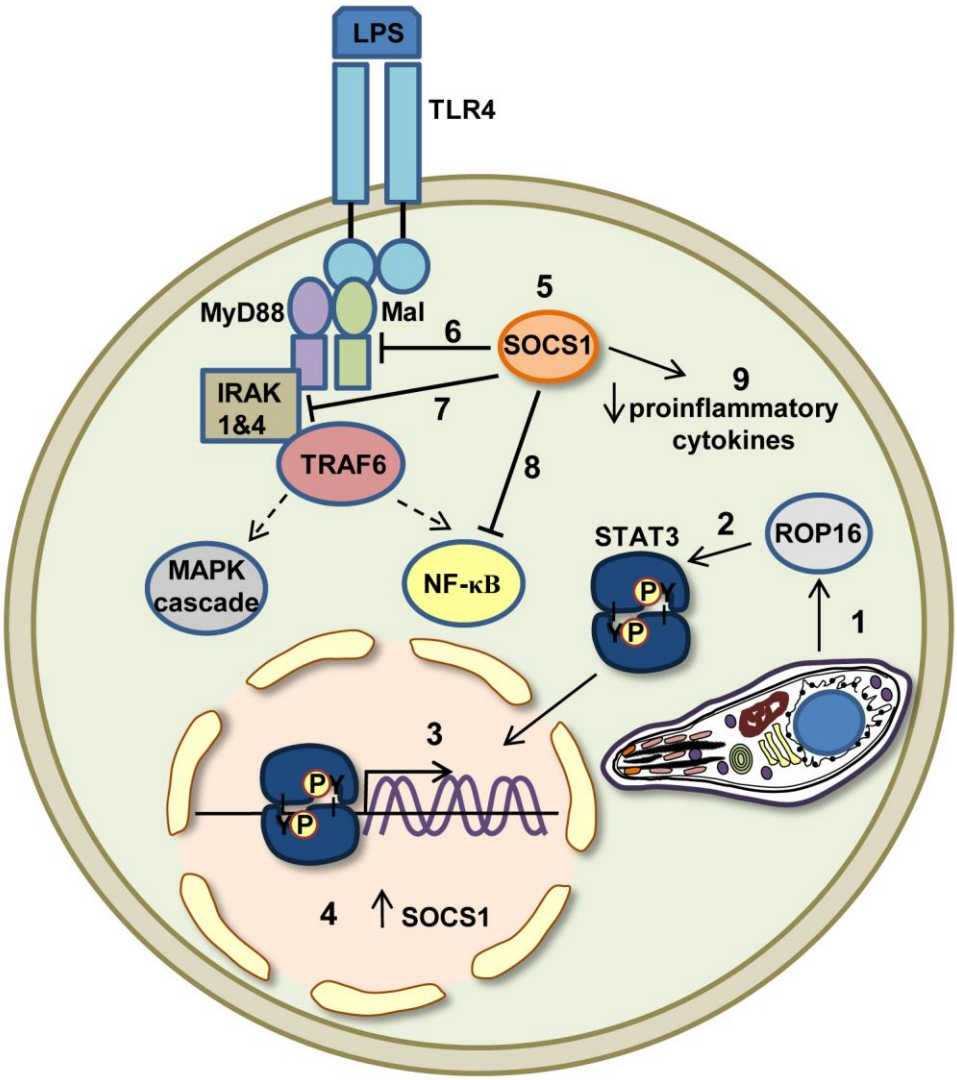

Figure 4.2. Proposed model for inhibition of TLR signaling by *Toxoplasma*-induced SOCS1. Upon host cell invasion, *Toxoplasma* secretes the rhoptry kinase ROP16 (1), which in turn phosphorylates host cell STAT3 (2). STAT3 translocates to the nucleus where it induces transcription of unknown genes in infected cells (3), which may include *Socs1* given its ROP16-dependent expression (4). SOCS1 is known to negatively regulate TLR pathways, with the TLR4 pathway provided as an example (5). Depending on the TLR or host cell involved, SOCS1 may target the adaptor protein Mal (6), the IRAK molecules (7), or NF-κB itself (8) for degradation. This in turn can affect activation or transactivation of NF-κB and/or activation of the MAPK cascade, leading to impaired pro-inflammatory cytokine production in *Toxoplasma*-infected cells subsequently exposed to TLR ligands (9).

However, this mechanism is unlikely to explain the initial inhibition of NFκB nuclear accumulation observed in macrophages infected with parasites alone (35), given the time needed for protein synthesis. Therefore, it is likely that the initial phase of NFκB inhibition occurs through a separate mechanism. Along these lines, it is known that in addition to ROP16, *Toxoplasma* can rapidly inject a serine-threonine phosphatase into the nucleus upon invasion (36). Presumably one of these molecules could alter the localization of NFκB early on, thereby preventing nuclear accumulation until the second phase mediated by STAT3 transcriptional activity could begin. Given that type II strains only induce NFκB activation after 4 hours or so (15), it is possible that the initial phase of NFκB activity is blocked equally by all parasite strains, permitting establishment of infection. Type I and III strains would then have the added advantage of further impairing proinflammatory cytokine production at later timepoints through the ROP16/STAT3/SOCS1 mechanism. This hypothesis could be tested initially by comparing proinflammatory cytokine production in infected cells in the presence or absence of SOCS1. *Socs1* knock-out mice do exist although they die in the first few weeks after birth (37). *Socs1*/IFNγ double knock-out mice survive longer and may be a better option for acquiring primary cells (38). If successful, further work could be done to confirm STAT3 involvement in *Socs1* induction and to confirm the negative impacts on TLR activity. It is also possible that *Toxoplasma*-induced STAT3 triggers expression of additional proteins that may also contribute to TLR regulation.

In summary, my work has shown that *Toxoplasma* can inhibit IFNγ/STAT1-dependent activity in the dendritic cell, a key cell type in the immune response to infection. Paradoxically, I observe that the parasite can also induce STAT1 phosphorylation independent of cytokine

stimulation. The parasite's impact on *Socs1* expression is complex, but may shed new light on how the parasite inhibits TLR-induced pro-inflammatory cytokine production. Further work as outlined above is required to further dissect the mechanisms by which these processes occur, as well as to determine what role *Toxoplasma*-activated STAT1 may play. Nonetheless, this work has contributed to our knowledge of how *Toxoplasma* successfully evades the host immune response.

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